Effects of environmental stress on gene expression in mussels

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Abstract

The biogeographic distribution of organisms is determined by physiological characteristics that enable a population to persist in a specific location. Global climate change effects are anticipated to increase the physiological stress experienced by organisms. Consequently, it is important to understand physiological responses to environmental stress and the mechanisms used by animals to cope with variable conditions.

I investigated the physiological response to environmental stress in two species of mussel from New Zealand, *Perna canaliculus* and *Mytilus galloprovincialis*, using quantitative PCR and ecological field experiments. A series of laboratory and field experiments were done to manipulate stress levels and the expression levels of three heat shock protein genes (*hsp24*, *hsp70*, *hsp90*) were measured. A transcription regulatory gene (*elf2*) and a cell cycle regulatory gene (*tis11d*) were also measured. The dynamics of stress response gene expression in response to acute stress and gene expression changes in the natural population due to varying forms of environmental stress were tested.

Between-zone translocations of different sized *M. galloprovincialis* and *P. canaliculus* were done at two sites in both east and west regions of the South Island of New Zealand. Site was found to be the most important factor in stress response. Apparent low food and high exposure stress interacted to create the particularly elevated stress response at the Timaru site. The adaptive ability of mussels transplanted between sites with varying environmental conditions was also tested. Results suggest that acclimation may be limited under stressful conditions. Furthermore, I found that *P. canaliculus,* the predominantly low-zone species, had a lower stress response than *M. galloprovincialis*, which was contradictory to predictions.

The investigations described in this thesis suggest that interactive effects of abiotic stress and food limitations are particularly challenging for animals. With the severity of climate change scenarios predicted, changes in water quality and aerial and seawater temperature suggest mussel populations are likely to be negatively affected in the future. This work also illustrates the great potential to utilise molecular techniques for analysis of physiological processes of non-model organisms in a real-world setting.

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Preface

A note about nomenclature.

Protein names are capitalised (Hsp70). Full gene names are lowercase and italic (*heat shock protein 70)* and gene symbols also lowercase and italic (*hsp70*). When a gene name appears at the beginning of a sentence, the first letter will be capitalised, but it will still appear in italics (*Hsp70*).

Translocation versus transplantation

Translocation refers to moving mussels within one site. Transplantation refers to moving mussels between sites.

Marine Collection Permit

The University of Canterbury has a special permit from the Ministry of Fisheries for the collection of marine specimens. All specimens collected for this thesis work fell under the permit. Special Permit (509), client number 8770058.

Chapter One: Introduction

Climate change and physiological stress response

1.1 Introduction

The mechanisms that determine the distribution of organisms has been a long-standing question in the field of ecology. This study aims to investigate the physiological tolerances of organisms in response to environmental stress by conducting field experiments and gene expression analysis on two species of mussel. Global air temperature has risen by 0.74˚C in the past century, with a linear warming trend for the past five decades (Trenberth *et al.*, 2007). There has been extensive melting of ice and snow cover in the northern hemisphere and global sea level is expected to rise approximately 4.2 mm $year^{-1}$ from the year 2000 to 2080 (IPCC, 2007). In addition, the pH of the oceans has dropped by 0.1 units since pre-industrial levels due to the absorption of CO_2 released into the atmosphere from anthropogenic sources (IPCC, 2007). These trends are predicted to continue, with the average global temperature and variability of storms predicted to increase, which will have unknown consequences to ecological communities (Christensen *et al.*, 2007).

The ability of animals to cope with increased environmental variability is of concern to biologists. Species that are unable to adapt to these environmental changes are at risk of range contraction and localised, or widespread, extinctions. It is likely that there will be significant changes in biogeographical distribution (Fuller *et al.*, 2010). The paragraphs below outline organismal responses (phenology, range shifts, species interactions and behaviour) to the effects of global climate change.

Changes in phenology and the distribution of organisms have already occurred as a consequence of climate change (Parmesan and Yohe, 2003). For example, the emergence date for butterflies (*Heteronympha merope*) in Melbourne has shifted an average of 1.5 days per decade over the past half-century, and this shift has been directly attributed to increased annual temperatures (Kearney *et al.*, 2010). Many species of bees have also shifted their phenologies. Over the past 100 years, North American bees are flying 10.4 ± 1.3 days earlier in the spring than before (Bartomeus *et al.*, 2011). The majority of this advancement in bee flight has occurred since 1970 and has been directly attributed to the effects of climate change (Bartomeus *et al.*, 2011). Many other examples of phenological shifts and their effects are available in the literature (e.g., early onset flowering in southern

Californian *Brassica rapa* (Franks *et al.*, 2007), and advanced egg laying dates in the Netherlands in the pied flycatcher *Ficedula hypoleuca* (Both and Visser, 2001)).

Some species are also showing evidence of range shifts in addition to changes in timing. Many terrestrial organisms are shifting poleward by approximately 6-25 km per decade (Parmesan and Yohe, 2003). Specifically, 12 bird species have extended their ranges northwards by an average of 18.9 km over the past 20 years in the United Kingdom (Thomas and Lennon, 1999). At multiple locations in the northern hemisphere, many species of *Lepidoptera* (butterflies and moths), and *Odonata* (dragonflies and damselflies) have shifted their ranges northwards or contracted their southern limits (reviewed in Parmesan, 2006).

In the marine realm, there is also evidence of range contraction and this tends to be much greater in magnitude compared to terrestrial biogeographical changes (Somero, 2011). For example, Jones *et al.* (2010; 2012) have shown a 350 km contraction of the southern limit of the barnacle *Semibalanus balanoides* over the past 50 years in the rocky intertidal zone of the eastern USA. Intertidal communities have changed on the Californian and British coasts, with a decrease in northern species and an increase in southern species (Southward *et al.*, 1995; Sagarin *et al.*, 1999). Pelagic species have also altered their distributions, with a shift of between 48 and 403 km in half of the fish species with range limits in the North Sea (Perry *et al.*, 2005). In 13 of 14 species whose ranges shifted, the range shifts were poleward, as predicted for climate-related responses. Additionally, copepod assemblages in the North Atlantic Ocean and European Shelf Seas have shifted northwards by greater than 1000 km in the past 50 years (Beaugrand *et al.*, 2002). Altogether, there is a growing body of evidence that species ranges are shifting, leading to changes in the composition of ecological communities around the world.

Biogeographic changes in the distribution of species can lead to changes in community structure with potential consequent effects on ecological relationships (Walther, 2010; Estes *et al.*, 2011). Species interactions are important to maintain community dynamics and stability within an ecosystem, particularly with respect to the interaction strength of predator-prey dynamics (Bascompte *et al.*, 2005; Tylianakis *et al.*, 2008; Estes *et al.*, 2011). Furthermore, within any particular community, certain members are more important to structure community dynamics. For example, apex predators exert strong influence on community

structure in many marine, terrestrial and freshwater aquatic ecosystems (Estes *et al.*, 2011). In the intertidal zone the keystone species concept is another example of how community structure is driven by the strong influences of particular species. The sea star, *Pisaster* sp., increases species diversity by preventing competitive dominance of mussels (Mytilus spp.; Paine, 1966; Jones *et al.*, 1994). Pollution, habitat loss and climate change are affecting communities by contributing to species range shifts (Estes *et al.*, 2011).

Global environmental change can result not only in phenological changes and range shifts, but can also affect the way in which species interact with each other in a normal community (Barton, 2010; Cripps *et al.*, 2011; Nowicki *et al.*, 2012; Pincebourde *et al.*, 2012). For example, the predation rate by the sea star *Pisaster ochraceous* is affected by aquatic and aerial thermal stress (Pincebourde *et al.*, 2012). Increased temporal variation of aquatic and aerial thermal stress events causes feeding behaviour of the sea star to decrease for several days, indicating that multiple stress events may lead to physiological vulnerability. Behavioural changes such as this suggest that as the climate becomes increasingly variable, there may be large changes in between species interactions. A further study shows that with increasing sea surface temperature and CO_2 concentration, the foraging behaviour of a Great Barrier Reef anemone fish *(Amphiprion melanopus)* changes (Nowicki *et al.*, 2012). Feeding decreases in higher temperatures or in higher temperatures with moderate CO_2 rise. However, feeding increases in higher temperatures with high CO_2 conditions. Another study shows that in high CO_2 conditions for *Pseudochromis fuscus*, a different Great Barrier Reef fish, predation behaviour (avoidance rather than attraction to injured prey) is inhibited by decreases in olfactory sensitivity (Cripps *et al.*, 2011). In terrestrial systems with elevated temperature, trophic interactions between predatory spiders and their grasshopper prey are affected through behavioural changes of both species (Barton, 2010). These studies show temperature and CO_2 increases associated with climate change are affecting predator/prey dynamics.

Evidence presented above outlines changes in phenology, range shifts, species interactions and behaviour that have all been linked to global climate change. However, there is a dearth of information about how specifically animals deal with the increased environmental variability and extremes associated with climate change. A central goal of ecological physiology is to understand how

organisms cope with stressful environmental conditions. Understanding how environmental conditions affect the physiology of an organism can allow predictions about which organisms may be at greater risk from the effects of global climate change. Understanding how animals deal with increased environmental variability and extremes is important to enable predictions and understanding of how communities might respond in future climate change scenarios (Somero, 2012).

1.2 Marine systems

Temperate organisms that experience fluctuations in their natural environment have the ability to respond physiologically to variable conditions. The extent to which an organism responds physiologically to stress depends greatly on the recent thermal conditions it has experienced (Hochachka and Somero, 2002). Induction temperatures of the heat shock response are elevated when organisms are adapted to warmer conditions. For example, mussels that are adapted to winter conditions initiate heat shock protein expression at 23˚C, whereas mussels adapted to summer conditions do not initiate expression until 28˚C (Buckley *et al.*, 2001; reviewed in Pörtner, 2002). The ability and extent of adaptation to changing thermal conditions is a response that occurs on many time scales, from minutes to multiple generations. Rapid response to thermal stress has been shown to occur in as few as 15 min of heat exposure in gill samples from *M. californianus* (Dutton and Hofmann, 2009). Alternatively, the same species sampled from a large geographical area (Baja California to Tatoosh Island) show differences in stress response induction temperatures after six weeks or longer in a common garden experiment (Logan *et al.*, 2012) suggesting that there may be population-specific genetic difference governing the heat shock response.

Mussels are important members of an ecosystem, as they are competitive dominants (Paine, 1974) and facilitators of diversity (Tsuchiya and Nishihira, 1986) on many rocky shores around the world (Seed and Suchanek, 1992; Morton, 2004). Within multilayered mussel beds there is less sunlight, lower temperatures, increased humidity and sediment compared to habitat outside the mussel bed (Suchanek, 1992). These conditions provide a home for one of the most diverse temperate communities of organisms worldwide (Tsuchiya and Nishihira, 1986; Suchanek, 1992). Therefore, shifts in the mussel community may have a large

impact on coastal ecosystems. Biogeographic shifts of mussels are also good indicators of climate change, as their biogeographic range limits tend to be well-studied and well-defined (Somero, 2012).

Intertidal marine organisms live in a delicate balance with the surrounding environment (Menge *et al.*, 2007). The intertidal zone is a physically challenging and variable habitat, making it ideal for the study of the organismal stress response because it encompasses both aquatic conditions during high tide and terrestrial conditions during low tide. Physical stressors there include large fluctuations in temperature, salinity, duration of aerial exposure and hydrodynamic forces (**Figure 1.1**). Vertical position in the intertidal zone, local tidal regimes and substrate angle can influence the intensity of these stressors (Helmuth and Hofmann, 2001) and as a result, different species are often vertically distributed in the intertidal (Helmuth *et al.*, 2006) (**Figure 1.2**). This vertical distribution results from the physiological tolerance of organisms to these and other abiotic factors and the biotic interactions among species (Dayton, 1971; Somero, 2002). The higher limits of a species' distribution are often set by physiological tolerance to abiotic conditions while the lower limits of a species' distribution can be set by the interaction among species in terms of competition for space and predation (Connell, 1961; Connell and Orias, 1964; Dayton, 1971; Menge, 1976).

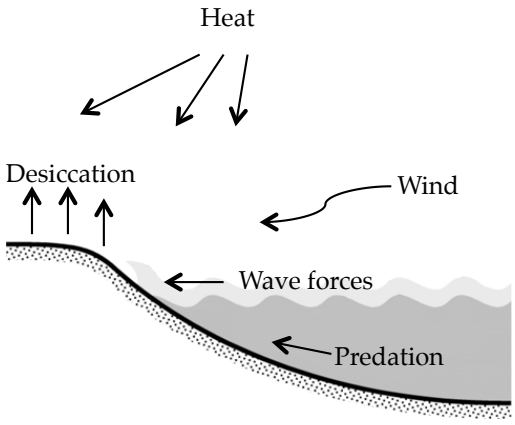


Figure 1.1 Some important abiotic factors that influence organisms living in the intertidal zone. Other biotic factors such as competition and facilitative processes also influence organisms within mussel beds.

Figure 1.2 The zonal dominance by various species is evident along Banks Peninsula. The low-zone (left of image) is dominated by the mussel *P. canaliculus* and the barnacle *Epopella plicata*. The mid-zone is dominated by the mussel *M. galloprovincialis* and the barnacle *Chamaesipho columna*. The high-zone (right of image) is dominated by the mussel *Limnoperna pulex*. Photo from Box Thumb on Banks Peninsula.

Animals that live in the upper limits of the intertidal zone often exist near their thermal maxima and are expected to be severely affected by climate change (Stillman, 2002; Stenseng *et al.*, 2005; Dong and Somero, 2009; Somero, 2012). For example, in California, the intertidal snail *Chlorostoma funebralis* (formerly of the genus *Tegula*) lives high in the intertidal region and is the most heat-tolerant among several congeneric species (*C. funebralis*, *C. brunnea* and *C. montereyi*) (Stenseng *et al.*, 2005). In snails acclimatised to 14˚C, the high intertidal zone species, *C. funebralis*, can withstand temperatures of up to 28.5˚C before reaching the Arrhenius break temperature (ABT). With increasing temperature, heart function increases to a point and then shows a sharp drop with additional temperature increases. The point where the heart function drops is considered the ABT. The low intertidal and subtidal species, *C. brunnea* and *C. montereyi* had ABT values of 20.2˚C and 21.7˚C respectively. Not surprisingly, the high intertidal zone species can withstand higher temperatures than the low intertidal zone species. Interestingly, when those three species are acclimated to 22˚C, the low and subtidal species were able to increase their ABT values by 6.6˚C and 4˚C to 26.8˚C

and 25.7˚C, while the high intertidal zone species increased its ABT value by only 1.6˚C to 30.1˚C. Twenty-two degrees is eight degrees above the normal environmental temperature for the low and subtidal species. The lower intertidal zone species have more acclimatory capacity and therefore are at a lower risk to the effects of climate change than the high intertidal zone snail species. Competition for space on the low shore may prevent these high-zone organisms from simply relocating down the shore (Hawkins *et al.*, 2008) and persistence of the population may be at risk.

Different mussel species in the genus *Mytilus* are model organisms for studying ecological dynamics and physiological responses to the environment (Paine, 1966; Kennedy, 1976; Menge *et al.*, 1994). The physiological response of *Mytilus* to many factors including, but not limited to, toxicology (Dondero *et al.*, 2006a), parasite load (Anestis *et al.*, 2010b) and thermal stress (Dutton and Hofmann, 2009; Ioannou *et al.*, 2009) have been studied. For instance, in farmed *M. galloprovincialis* from the Mediterranean Sea, the inducible heat shock response is turned on in the seasonal transition from winter to spring. As the water temperature increases at the end of winter, the mussels are not acclimated to the warmer temperature and therefore need to counteract the thermal stress that they experience (Ioannou *et al.*, 2009). Additionally, pyruvate kinase activity (a key glycolytic enzyme) increases in the seasonal transition period as well, indicating that there are increased metabolic demands attributable to the heat shock response (Ioannou *et al.*, 2009). If the heat shock response is normally initiated during seasonal transitions, then populations may be vulnerable to additional stress associated with global climate change conditions. The specifics of heat shock responses are presented in **Section 1.3**.

The genus *Mytilus* includes four species of mussels, *M. edulis, M. trossulus, M. californianus* and *M. galloprovincialis* (Seed, 1992). *M. trossulus* is the ancestral species, which lives on the west coast of North America and diverged ~3.5 million years ago into *M. edulis* (Riginos *et al.*, 2004). *M. edulis* is native to the North Atlantic Ocean and a speciation event about two million years ago led to the existence of *M. galloprovincialis,* a warm-adapted mussel native to the Mediterranean Sea (Seed, 1992). The physiology of *M. galloprovincialis* has helped it spread across the world as one of the most successful invasive species on the planet (Fields *et al.*, 2006; Somero, 2012). *M. galloprovincialis* has been present in

New Zealand for over one million years (Gardner, 2004). It can currently be found the length of New Zealand where there is appropriate substrate from 32˚ S to 52˚ S including the off-shore islands (Morton, 2004). There is limited information about the phylogenetic structure of the New Zealand populations of *M. galloprovincialis.*

The evolutionary origin of *M. galloprovincialis* populations in the entire southern hemisphere, including samples from seven populations on the South Island, was investigated in Westfall and Gardner (2010). The South Island populations were confirmed as *M. galloprovincialis* but no detail about the phylogenetic population structure was included in their study. The genetic population structure of *M. galloprovincialis* in the far north of New Zealand was investigated by Gardner and Westfall (2012) and they found evidence of invasion of *M. galloprovincialis* from the Northern hemisphere. Neither of these studies provides any information about the phylogenetic population structure of South Island populations of *M. galloprovincialis* used in this thesis.

Perna is another important genus in the family Mytilideae. Although not as widespread as M*ytilus* spp., the genus *Perna* tends to be predominantly a southern hemisphere taxon (Siddall, 1980; Wood *et al.*, 2007). *P. viridis* is native to the Indo-Pacific, *P. perna* is found around Africa and South America and *P. canaliculus* is present exclusively in New Zealand (Vakily, 1989; Wood *et al.*, 2007). *P. canaliculus* is competitively dominant in the low intertidal zone in New Zealand (Paine, 1971), outcompeting *M. galloprovincialis* (Menge *et al.*, 1999; Morton, 2004). *P. canaliculus* is an important aquaculture species in New Zealand, with an estimated export revenue of NZ$ 220.5 million in 2011 (Aquaculture New Zealand, 2012). Genetically, there is a break in the population between the North and South Islands (Apte and Gardner, 2002; Star *et al.*, 2003), with populations on the west coast of the South Island being the most distinct from the northern populations (Star *et al.*, 2003). The *P. canaliculus* population at Timaru, a site on the east coast of the South Island, has the South Island haplotype but clusters with the North Island populations (Apte and Gardner, 2002). Timaru has a shipping port and may have mussels that introduced from the North Island. Together, *M. galloprovincialis* and *P. canaliculus* are important ecosystem engineers on the New Zealand rocky shore in the mid to low intertidal zone (**Figure 1.3**) because they provide habitat for many species and are dominant in the zones they occupy (Morton, 2004).

Figure 1.3 Image of *M. galloprovincialis* (left) and *P. canaliculus* (right).

In New Zealand rocky shore habitats where mussels dominate, *P. canaliculus* inhabits the subtidal and low intertidal zone and *M. galloprovincialis* occupies mostly the mid intertidal zone (Morton, 2004). The boundary between the two species is somewhat indistinct in the New Zealand rocky intertidal, with an area where both species co-occur (Menge *et al.*, 2007). Intertidal zonation patterns are sometimes produced from the results of interspecific competition where the presence of one species prevents the persistence of another species. For example, Dayton (1971) showed that vertical zonation of the mussel *M. californianus* in the low-zone and the barnacle *Balanus glandula* in the mid-zone was the result of the mussel overgrowing and smothering the barnacle up to a certain shore height. Menge (1976) showed that juveniles of the mussel *M. edulis* in the low-zone were able to settle upon and then overgrow and smother the barnacle *Semibalanus balanoides*. In the region above where *M. edulis* grew, the barnacle was able to persist because the mussel was either unable to survive at that height or juveniles were unable to recruit at that shore height (Menge, 1976). In a barnacle-barnacle interaction on the coast of Scotland, Connell (1961) showed that the high-zone species *Chthamalus stellatus* was excluded from the lower-zone due to crushing from the low-zone dominant species *S. balanoides.* In New Zealand, the distinction between zones is not as clear as in these other examples described above. As mentioned, *P. canaliculus* and *M. galloprovincialis* overlap, which indicates the zonation is unlikely to be caused directly by interspecific competition between the two species. Instead, there is likely to be some differences in the physiological tolerances between the two species. *P. canaliculus* is more abundant in wave exposed areas while *M. galloprovincialis* is more abundant in wave protected areas (Menge *et al.*, 1999). Indeed, the concept of differences between the physiological tolerances of the two species was investigated by Menge *et al.* (2007). The

experimental design included 21 predictions of the effects of site, zone and magnitude of wave exposure on *P. canaliculus* and *M. galloprovincialis.* To summarise the predictions, based on the natural distribution of the two species, *M. galloprovincialis* was expected to perform best in the mid-zone at the wave-sheltered site, while *P. canaliculus* was expected to perform best in the low-zone of the wave-exposed site (Morton, 2004; Menge *et al.*, 2007). Of these 21 predictions, only 12 were supported while the other nine were not (**Table 1.1**). The factors assessed were: survival, growth, relative tissue mass and RNA:DNA ratio. The RNA:DNA ratio is a measure that is used to assess the rate of protein synthesis, which is an index of short-term growth capacity (Dahlhoff *et al.*, 2002; Menge *et al.*, 2007). Due to the limited support in the results for their hypotheses, they recommended additional study of physiological responses in *M. galloprovincialis* and *P. canaliculus*. In another study, it was shown that a summer heat wave resulted in the death of 35.4% of *P. canaliculus* living in the area where the two specie co-occur while only 3.4% of *M. galloprovincialis* in the same region (Petes *et al.*, 2007). These results suggest that *P. canaliculus* is less thermally tolerant than *M. galloprovincialis*.

Table 1.1 To illustrate the results of ecological (mortality rate, growth rate and relative tissue mass) and physiological responses (RNA:DNA ratio) in *P. canaliculus* and *M. galloprovincialis,* this table is from Menge *et al.* (2007). "…Summary of results, comparing predicted or expected effects with observed effects of different factors (site, zone) on the measures of performance of mussels. Bold indicates observed results that were consistent with expectations. BB: Boulder Bay; BT: Box Thumb; M: mid; L: low." For example, in the first line of the table, the mortality rate of *P. canaliculus* is expected to be smaller at BB than at BT in both intertidal zones. However, the observed data showed that mortality rate was equal in the mid-zone at both sites and followed the predictions in the low-zone.

Measure	Species	Site			Zone		
		Expected	Zone	Observed	Expected	Site	Observed
Mortality rate	*Perna*	BB<BT	Mid	BB=BT	M>L	BB	**M>L**
			Low	**BB<BT**		BT	M=L
	Mytilus	BB≥BT	Mid	**BB=BT**	M>L	Both	**M>L**
			Low	BB<BT			
Growth rate	*Perna*	BB>BT	Mid	BB<BT	M<L	Both	**M<L**
			Low	BB≤BT			
	Mytilus	BB≤BT	Mid	**BB<BT**	M<L	Both	**M<L**
Relative tissue mass	*Perna*	BB>BT	Mid	BB<BT	M<L	Both	**M<L**
			Low	BB≤BT			
	Mytilus	BB≤BT	Both	**BB<BT**	M<L	Both	**M<L**
RNA:DNA	*Perna*	BB>BT	Both	**BB>BT**	M<L	Both	M=L
	Mytilus	BB≤BT	Both	**BB<BT**	M<L	Both	M=L

1.3 Heat shock response

'Stress' has many definitions depending on the context. For the purposes of this study, an animal is 'stressed' when the physiological or chemical environment is beyond the normal range and the regular function of cells or proteins is disturbed to such an extent that survival is reduced (Selye, 1950). Stress on organisms, including mussels, in the intertidal zone is known to affect their biogeographic distribution (Helmuth and Hofmann, 2001). Physiological responses are required to counteract deleterious effects of stress-inducing factors, thus prolonging survival. Cells often respond to stress through changes in gene expression that result in the upregulation of a highly conserved superfamily of proteins, the heat shock proteins (Hsps). Hsps are one of the most well-studied groups of physiological adaptation mechanisms (Lindquist, 1986; Pörtner, 2002).

They were first identified in 1960 by Ritossa (1962) who was studying the effect of heat shock on *Drosophila.* It was noticed that the chromosomes in salivary glands would 'puff' after heat shock. These swellings were attributed to the activation of genes in that area of the chromosome. Proteins arising from these chromosome puffs became known as heat shock proteins and it is now known that heat shock proteins are found in all plants and animals (Ritossa, 1996; Feder and Hofmann, 1999). Their regulation in response to stress has been studied in a wide variety of organisms (Tomanek, 2010) and their upregulation is induced by stress resulting from a wide variety of factors. In addition to thermal stress, hypoxia, acidosis, toxins, osmotic shock, microbial damage and even crowding can cause upregulation of Hsps (reviewed in Feder and Hofmann, 1999; Roberts *et al.*, 2010).

Hsps function as molecular chaperone proteins that interact with the folding of nascent polypeptides and in the maintenance of normal protein function in cells (Feder and Hofmann, 1999). For optimal protein function, there is a delicate balance between temperature and protein stability, especially if a specific conformational structure is required at the binding site for proteins to function in the most efficient manner (Fields, 2001). Many intermolecular interactions, such as salt bridges, hydrophobic interactions and hydrogen bonds, maintain the specific three-dimensional structure (Fields, 2001). Many different conditions affect the conformational structure of the protein and thereby affect the catalytic rate, or k_{cat}, of enzymatic function (**Figure 1.4**). k_{cat} functions optimally at certain temperatures with a high rate of enzyme catalysis. If temperature increases beyond a certain point the turnover rate will plummet, indicating the localised denaturation of a protein's active site (Licht, 1964; Fields, 2001; Somero, 2011). If temperature increases further, the protein is likely to be denatured completely and entirely broken down by proteolytic enzymes.

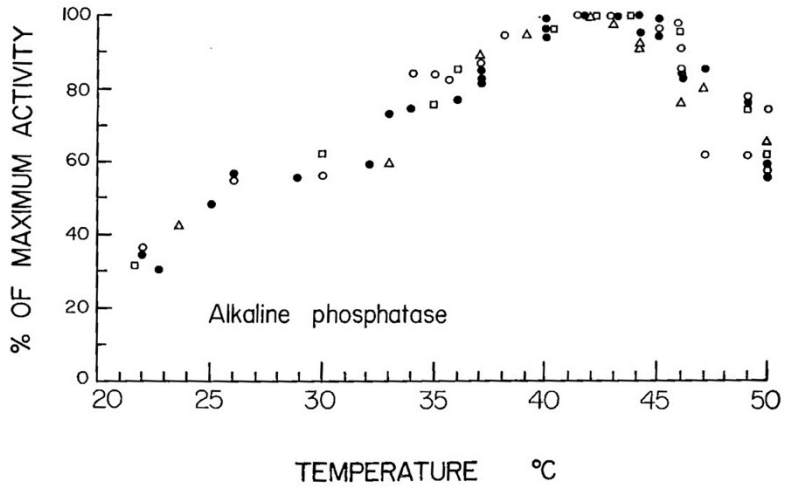


Figure 1.4 As temperature increases, protein activity increases, reaches a maximum, then decreases. Figure adapted from Licht (1964). Influence of temperature on alkaline phosphatase activity in lizards.

Many abiotic factors can affect the integrity of the protein pool within cells, which in turn affects the cellular functions required for normal processes to occur. The exposure of hydrophobic regions of the protein elicits a cellular stress response when proteins begin to denature, which functions to prevent further damage and to repair existing structural damage (Tomanek, 2010). The cellular stress response is well-characterised and conserved among diverse taxa. It is used to cope with a multitude of environmental stressors (Feder and Hofmann, 1999). Expression of chaperone proteins such as Hsps is upregulated and used to repair the damage (Tomanek, 2010). The amount of Hsps present in a cell is an excellent indicator of current stress levels (Feder and Hofmann, 1999). There are several classes of Hsp genes that are grouped by protein size: *hsp110, hsp100, hsp90, hsp70, hsp60, hsp40, hsp10* and the small *hsp* families (Lindquist, 1986; Gething, 1997; Feder and Hofmann, 1999). For the purposes of this thesis, the focus will be on the small *hsp, hsp24,* as well as *hsp70* and *hsp90*, discussed further in Chapter 2.

Members of the small *hsp* (*shsp*) gene family range in size from 16 to 42 kDa. They are present in both plants and animals, but the number of *shsp* members differs substantially among taxa (Lindquist, 1986; Heikkila, 2010). SHsps are more species-specific than other Hsps. Sequence homology of *shsps* is highly divergent between taxa except for in the c-terminal region, which has a highly conserved α-

crystallin domain (de Jong *et al.*, 1993). SHsps are generally not found in the cell except in response to stress and are known for their rapid upregulation (Roberts *et al.*, 2010). Heat-induced aggregation of damaged proteins can be lethal for the cell. However, sHsps can prevent this by stabilising damaged proteins in a folding-competent state.

Damaged proteins can be refolded back into a native conformation by other chaperone proteins such as Hsp70 or Hsp90 (de Jong *et al.*, 1993; Heikkila, 2010; Tomanek, 2010). SHsps can inhibit programmed cell death by down-regulating caspase activity (Beere, 2004) and are important for actin dynamics in cytoskeletal stability (Heikkila, 2010).

Hsp70 and *hsp90* are synthesised more slowly than *shsps* and they function to refold damaged proteins, allowing the restoration of protein function (Podrabsky and Somero, 2004; Tomanek, 2010). The *hsp70* and *hsp90* gene families often work together in a more sustained manner relative to the *shsps* (Tomanek, 2010). The *hsp70* family is the largest and most highly conserved Hsp family (Lindquist, 1986; Feder and Hofmann, 1999) and is well-characterised in the literature. Hsp70 is mainly found in the cytoplasm, but there are several members including constitutively active heat shock cognate *hsc70*, and members resident to the mitochondria and the endoplasmic reticulum. These molecular chaperones protect and repair nascent or denatured polypeptides, assist refolding to native conformation and prevent aggregation and programmed cell death (apoptosis; Beere, 2004; reviewed in Heikkila, 2010). *Hsp70* is upregulated when the cell comes under stress and native proteins begin to exhibit deformations (Tomanek, 2010).

Hsp90 is constitutively expressed in unstressed animals but upregulated during thermal acclimatisation and more so in times of stress (Heikkila, 2010). *Hsp70* and *hsp90* are elevated with chronic stress while *shsps* are more responsive to short-term stress (Tomanek, 2010). If stress experienced by a cell is too drastic, cells are degraded; Hsp90 is involved in apoptosis (Roberts *et al.*, 2010).

In addition to hsps, numerous other genes and gene families are involved in the heat shock response. Examples include *E74-like factor 2* (*elf*2) and *butyrate response factor 2* (*tis11d*), which are upregulated in stressed populations of *Mytilus californianus* (Place *et al.*, 2008). *Elf*2 is an ETS domain transcription factor. ETS-domain family members are found in all metazoans and have diverse biological roles (Sharrocks, 2001). Mammalian cancer studies involve *elf*2 in the regulation of

valosin-containing-protein, a molecule involved in angiogenesis and cell survival under cytokine stress (Zhang *et al.*, 2007). Endothelial cells upregulate *elf2* in response to hypoxia (Christensen *et al.*, 2002). Whether or not the upregulation of *elf2* in *M. californianus* is related to hypoxia or cytokine stress is unknown.

Tis11d is a destabilising protein that is involved in the post-transcriptional regulation of numerous genes (Hudson *et al.*, 2004; Baou *et al.*, 2009). It binds to adenine-uridine rich elements (AREs) and promotes deadenylation, decapping and degradation of mRNAs preventing protein expression. Many cytokines, transcription factors, cell cycle regulators, and apoptosis regulators contain AREs, thus Tis11d has many targets (Baou *et al.*, 2009). Heat shock stress induces Tis11d activity to enhance degradation of ARE-containing mRNAs (Murata *et al.*, 2005).

Studies on stress response can be done in the laboratory or in the natural environment using field experiments. Controlled experimental ecosystems or laboratory studies, also known as microcosms, mesocosms or microecosystems are often used to study biological questions (Chen *et al.*, 2000; Petersen *et al.*, 2003). For clarity, mesocosm will be used to refer to all these types of experiments. These controlled systems have become widely used tools in ecological research (Ives *et al.*, 1996). Some stress response gene expression studies have been done in mussels to assess responses to environmental stress in laboratory mesocosms (Dondero *et al.*, 2005; Dondero *et al.*, 2006a; Dondero *et al.*, 2006b; Anestis *et al.*, 2007; Anestis *et al.*, 2008; Anestis *et al.*, 2010b; Anestis *et al.*, 2010a; Lockwood *et al.*, 2010) or in the field (Dondero *et al.*, 2006a; Dondero *et al.*, 2006b; Gracey *et al.*, 2008; Jones *et al.*, 2010; Connor and Gracey, 2011; Núñez-Acuña *et al.*, 2012; Place *et al.*, 2012). Mesocosm studies are beneficial in that the degree of control, replication and repeatability is high (Ives *et al.*, 1996). A mesocosm can be used to test specific predictions based on theoretical models in a 'clean' system (Daehler and Strong, 1996). The degree of control available in mesocosm studies is far beyond that which can be achieved in a natural setting where the influence of a multitude of factors such as weather patterns, nutrient levels and inter/intra specific competition are difficult to control or even measure (Odum, 1984; Petersen *et al.*, 2003). Using mesocosms, some studies can be done that would otherwise not be possible. For instance, Jaffee (1996) describes a series of studies in soil systems that examine the interactions between nematodes and parasitic fungi. Due to the nature of soil ecosystems, studying nematodes in the natural environment is not

feasible. The organisms are so small that a population can only be sampled once through destructive methods, soil can be heterogeneous which results in adjacent patches likely having different dynamics (e.g., number of plant roots in a patch) and soil is opaque which makes observations of the system impossible (Jaffee, 1996). Using mesocosms, the interactions of the nematodes and fungi can be studied, controlled and manipulated to address the research questions that would otherwise be untestable.

Mesocosms are often criticised for representing unreal conditions. Some of the advantages of mesocosms come at a cost when effects are translated to natural situations. For instance, their small size, often short duration and their simplified biological and physical complexity can result in difficulties extrapolating the results out to be applicable to real-world ecosystems (Petersen *et al.*, 2003). It is very difficult to recreate the true natural conditions in a mesocosm (e.g., nutrient levels, microclimate, species assemblages, etc.) which calls into question how relevant mesocosm results actually are to true communities (Petersen *et al.*, 2003). Additionally, there can be large tank-effects which can bias the results be altering the organisms' response to a treatment (Petersen and Englund, 2005). Small-scale mesocosm studies can provide results that do not truly represent what occurs in a real-world setting. For instance, Stachowicz *et al.* (2008) have shown that biomass accumulation is positively associated with species richness in field experiments. However, in mesocosms, species richness has no effect at all. Biomass accumulation in mesocosm resulted solely through species identity. As a result, care must be taken when extrapolating results from mesocosm studies.

Field experiments do represent real-world scenarios but can be difficult to control and therefore results can become too complex for interpretation (Odum, 1984; Petersen and Englund, 2005). Despite the difficulty with field experiments they are important as they represent reality and encompass natural variation in the system. Ideally, using multiple experimental approaches for one biological question could provide the best scenario for testing hypothesis (Odum, 1984; Petersen and Englund, 2005). Using the controlled mesocosm, specific questions can be addressed, while in the field, more broad-scale tests could be done. If the results are congruent with each other, more weight can be put on the conclusions drawn about the phenomena being investigated.

Generally, mussel mesocosm studies consist of animals in seawater tanks and variables such as temperature, CO_2 or nutrients/toxins are manipulated (Dondero *et al.*, 2005; Dondero *et al.*, 2006a; Dondero *et al.*, 2006b; Anestis *et al.*, 2007; Anestis *et al.*, 2008; Anestis *et al.*, 2010b; Anestis *et al.*, 2010a; Lockwood *et al.*, 2010). Following experimental manipulations in laboratory mesocosms, the organisms' response to a particular variable is assessed. Studies that have been done in the field often have little to no experimentally manipulative component to the study. For example, a study done to assess the effect of heavy metal contamination on gene expression in *M. galloprovincialis* and *M. edulis*, showed that when mussels were exposed to heavy metals, the expression of metallothioneins (proteins that bind heavy metals) is upregulated (Dondero *et al.*, 2005). Mesocosm studies are beneficial for assessing physiological responses to specific environmental variables, but they do not provide real-world information about how an organism will respond in the natural environment. Subsequent to the mesocosm studies by Dondero *et al.* (2005), Dondero *et al.* (2006b) studied the effects of heavy metals at various field locations near a copper mine in Norway. The expression of stress response genes decreased at varying distances away from the mine (decreasing levels of contamination), showing that the intensity of the molecular markers, as indicators of physiological response was linked to the degree of contamination (Dondero *et al.*, 2006b). The progression from lab-based studies to field experiments is a good way to assess the effects of environmental stress, as it is important to have a more complete picture of the environment in which the organism actually lives.

Lockwood *et al.* (2010) performed acute heat stress experiments on the native *M. trossulus* and the invasive *M. galloprovincialis* in mesocosms and compared transcriptome expression between the two species. They found distinct differences in gene expression patterns between them. Notable differences in gene expression between the two species included the upregulated expression of *hsp24* and *hsp90* in *M. trossulus*. The gene expression patterns suggest that *M. galloprovincialis* will be more able in future climate change scenarios to gain a stronger foothold in the habitats they are invading. Enhanced stress response from the native species, *M. trossulus*, suggests that that species is more at risk to thermal stress than the invading species.

Several other mesocosm studies have investigated the effect of mercury (Dondero *et al.*, 2006a), thermal stress (Anestis *et al.*, 2007; 2008), temperature and hypoxia (Anestis *et al.*, 2010a) or temperature and parasite load (Anestis *et al.*, 2010b) on the heat shock response and metabolic activity.

Hsp70 protein expression has been studied through time in the field. Jones *et al.* (2010) found that hsp70 expression was inducible at temperatures of 32˚C and the periods of increased hsp70 expression coincided with high mortality events in periods of sustained high temperature. Jones *et al.* (2010) concluded that populations of *M. edulis* may be at risk in their study location on the east coast of North America. Connor and Gracey (2011) and Gracey *et al.* (2008) studied cyclical variations in gene expression in mesocosms and the natural environment. Using a microarray, they showed that during normal tidal oscillations the expression of many genes was rhythmical. Variation in gene expression between-sites and between-individuals within sites has been studied (Núñez-Acuña *et al.*, 2012; Place *et al.*, 2012). These studies have shown that there is considerable variation between sites but reasonably low variation between individuals within one site. This has positive implications for experimental design, such that the variation between individuals at a particular site is low, and so smaller sample sizes can be used to make inferences about population gene expression levels.

There have been a few field experiments where mussels were collected from different field sites and assessed for their physiological state. These studies provide a snapshot of the state of the organisms. Place *et al.* (2008) collected *M. californianus* from four sites on the west coast of North America spanning several degrees of latitude. They used a microarray and compared the transcriptome among sites. Many stress response genes (including *hsp70*, *elf2* and *tis11d*) were significantly upregulated at the more environmentally stressful site relative to the expression at more benign sites.

Experiments that are done in a natural setting with manipulative characteristics are still in short supply. To further understand the effect of environmental stress levels and physiological responses, manipulation of stress levels in the field would be a significant addition to our understanding of how animals respond to environmental stress in a real-world setting.

1.4 New Zealand oceanography

As a background to the selection of study sites (**Section 1.5**) it is important to have a picture of the varying oceanographic climate. New Zealand is a coastal nation with 18 000 km of coastal territory in the form of inlets, harbours, bays, estuaries and open coasts. The country spans 13˚ of latitude and ranges from subtropic to subantarctic conditions (Hart and Bryan, 2008). There are significant differences in oceanographic conditions between coasts on the South Island, with predominant downwelling on the east coast and upwelling on the west coast (Menge *et al.*, 2003). The prevalence of west coast upwelling dynamics have been called into question by Schiel (2004) who postulates that the upwelling-like conditions may be the result of heavy rainfall and riverine inputs rather than deep-water upwelling. Either way, the west coast marine communities are more productive than those on the east coast (Menge *et al.*, 2003; Menge *et al.*, 2007; Bracken *et al.*, 2012). As a result of the different oceanographic conditions on the opposite coasts, the east coast tends to be nutrient poor with less intense grazing, fewer large predators, lower predation rates and lower invertebrate recruitment (Menge *et al.*, 2003; Bracken *et al.*, 2012). In Pegasus Bay, to the north of the Banks Peninsula on the east coast, there is a persistent eddy that traps nutrients and phytoplankton, providing populations in that region with higher levels of particulate food compared to other east coast regions of the South Island (Menge *et al.*, 1999; Menge *et al.*, 2003).

1.5 Field Sites

The field sites in this study were all located on the South Island of New Zealand (**Figure 1.5**). There were two sites on the west coast (Woodpecker Bay and Nine Mile Beach) and five sites on the east coast (Raramai, Cave Rock, Box Thumb, Timaru and Shag Point). The locations of the sites are summarised in **Table 1.2** and the following sections describe the characteristics of each site.

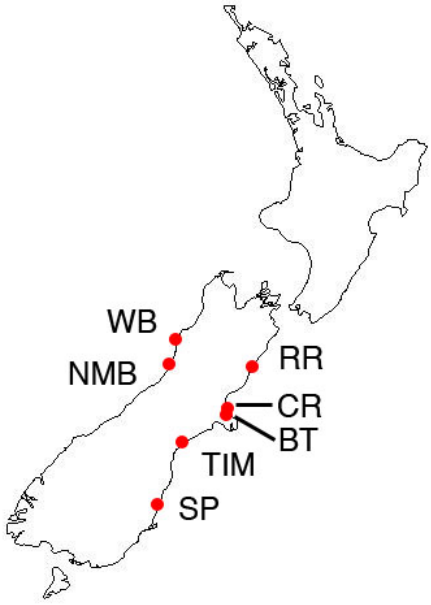


Figure 1.5 Map of New Zealand field sites. Site name abbreviations can be found in **Table 1.2**.

Table 1.2 Summary table of field sites.

Site Name	Abbreviation	Coast	Location
Woodpecker Bay	WB	West	42° 01.914' S, 171° 22.682' E
Nine Mile Beach	NMB	West	42° 20.996' S, 171° 15.166' E
Raramai	RR	East	42° 27.570′ S, 173° 33.043′ E
Cave Rock	CR	East	43° 33.896′ S, 172° 45.608′ E
Box Thumb	BT	East	43° 35.062′ S, 172° 47.409′ E
Timaru	TIM	East	44° 22.374' S, 171° 15.212' E
Shag Point	SP	East	45° 28.354′ S, 170° 49.909′ E

1.5.1 New Zealand intertidal zone

At most of the sites, the low intertidal zone is dominated by the green-lipped mussel *P. canaliculus* and the macroalgae *Durvillaea antarctica* and *D. willana*. The mid intertidal zone is dominated mainly by the blue mussel *M. galloprovincialis* with some *P. canaliculus* interspersed. The high intertidal zone is mainly the small black mussel *Limnoperna pulex* (formerly *Xenostrobus pulex*) as well as various barnacle species.

West coast

Woodpecker Bay

Woodpecker Bay (WB) is situated on the northwest coast of the South Island of New Zealand (**Figure 1.6**). Benches at WB are composed of hard metamorphic rock and are considered very exposed (Morton, 2004; Rilov and Schiel, 2006b). The site is made up of several benches of different sizes separated by areas of sand. There is extensive mussel and barnacle cover on the rocks. The sessile invertebrates are replaced mainly by large *Durvillaea* spp. in the lower shore level.

Figure 1.6 Woodpecker Bay, west coast, New Zealand.

Nine Mile Beach

Nine Mile Beach (NMB) is situated approximately 15 km north of the west coast mining community of Greymouth, and 50 km south of WB (**Figure 1.7**). The intertidal region at NMB is a flat rocky point at the south end of a long gravel beach. It is gently sloping to the water and at the exposed end of the bench the rocks end abruptly dropping vertically into the sea. There are mussel beds covering much of the bench, while the lowest part of the reef is dominated by red algae (Rilov and Schiel, 2006b). There are several surge channels that cut far into the bench, providing refuge for the sea star *Stichaster australis*.

Figure 1.7 Nine Mile Beach, west coast, New Zealand.

East Coast

Raramai

Raramai (RR) is on the east coast of the South Island approximately 10 km south of Kaikoura (**Figure 1.8**). The site is one of several rocky points in the area separated by gravel beaches. The bench has a complex topography and multiple channels that run through it. RR faces south and the mid-intertidal zone is mainly dominated by barnacles. There is a small population of mussels living in cracks and crevices, and the lower intertidal zone is dominated by *Durvillaea* spp.

Figure 1.8 Raramai, east coast, New Zealand.

Cave Rock

Cave Rock (CR) is near a popular beach in Sumner, a suburb of Christchurch (**Figure 1.9**). It consists of rock outcroppings that jut from the sand. The rocks used for the experiments in this thesis were about 6 m x 12 m and rose about 2 m above the sand. Mussels dominate the rocks and there is little algae present, except for ephemeral *Ulva* spp. that was generally present only after a disturbance cleared some rocky surface creating new habitat. During the course of the experiments at CR, there was considerable sand movement. Approximately one month after the 2009 experiment began, about 50 cm of sand had come in and covered part of the CR rocks. About two months later, the level of sand was almost 1 m less than at the start, exposing a great amount of bare rock that had previously been deeply buried (**Figure 1.9** inset).

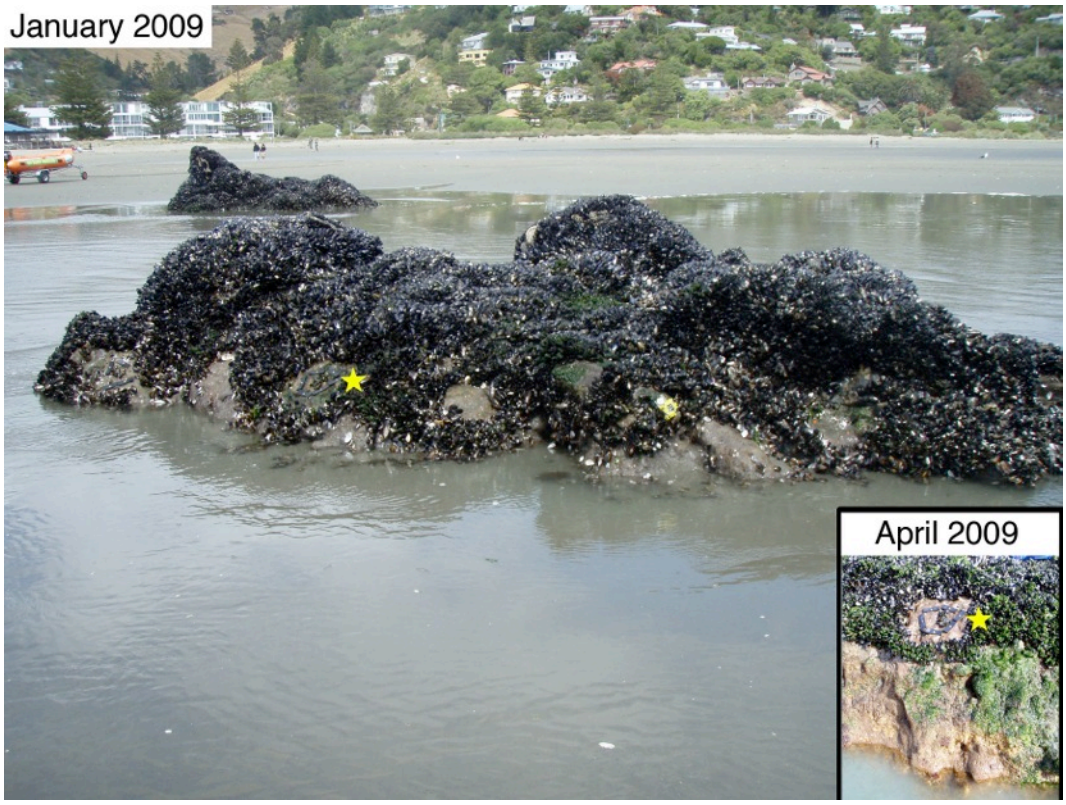


Figure 1.9 Cave Rock, east coast, New Zealand. Inset is the same rock from a few months later when sand had moved away from the rocks exposing bare rock. The star (yellow in colour photograph) shows the same location in both photos.

Box Thumb

Box Thumb (BT) is a northeast facing small peninsula situated on the north side of Godley Head, just south of Christchurch (**Figure 1.10**). BT is a rocky platform with a basaltic substratum and simple topography. Dense mussel and barnacle beds dominate the mid intertidal zone, and the low intertidal zone is dominated by brown algae *Durvillaea* spp. and *Undaria pinnatifida*.

Figure 1.10 Box Thumb, east coast, New Zealand.

Timaru

Timaru (TIM) is a city on the east coast, 160 km south of Christchurch. On the north side of the city, there is a boulder bench surrounded by sandy beaches (**Figure 1.11**). At the exposed edge of the bench, there is a sharp drop into deeper water that makes the site quite exposed. The sides of most boulders are covered with mussels in the mid intertidal zone, and in the low-zone mussels and macroalgae dominate.

Figure 1.11 Timaru, east coast, New Zealand.

Shag Point

Shag Point (SP) is 300 km south of Christchurch on the east coast with a mudstone bench (**Figure 1.12**). There are very few mussels or barnacles at SP, with tide pools in the mid to high intertidal zone occupied by motile invertebrates and turf algae. Macroalgae dominate the low intertidal zone.

Figure 1.12 Shag Point, east coast, New Zealand.

1.6 Aims and objectives

The aim of this study was to assess physiological stress on intertidal organisms through gene expression responses, growth and survival. This research also aimed to develop molecular techniques for the assessment of the extent of stress on different species of mussels. The molecular analyses were then used to test stress responses of mussels that had undergone different intensities/types of stress. The mussel stress tests were done in laboratory mesocosm studies as well as in field settings. Taken together, these laboratory and field-based studies were meant to define responses and to see the extent to which they operated in heterogeneous field conditions.

In Chapter Two, descriptions of the development of molecular techniques for use in mussels are provided. Stress response gene discovery for *M. galloprovincialis* and *P. canaliculus* is done and primers and quantitative PCR were developed and optimised.

In Chapter Three, the effects of acute thermal stress in *M. galloprovincialis* on gene expression of the genes identified in Chapter Two are tested. The timing, duration and extent of stress response gene expression are studied.
Chapter Three hypotheses:

- Gene expression of stress response genes is upregulated when mussels are exposed to acute thermal stress;
- Speed and intensity of gene expression changes will be different for different stress response genes. *Hsp24* will respond rapidly to increased temperatures, *hsp70* and *hsp90* will respond more slowly than *hsp24* but more quickly than *tis11d* and *elf2.*

In Chapter Four, how mussels respond to changes in their local environment is tested by translocating mussels between intertidal zones at several sites around the South Island.
Chapter Four hypotheses:

- Mussels translocated from their native intertidal zone into a different intertidal zone will show evidence of stress over the course of summer;

- Small mussels will have an enhanced stress response relative to large mussels;
- *M. galloprovincialis* (a true intertidal species) will have a lower intensity of stress response than *P. canaliculus* (a low intertidal-subtidal species);
- There will be a higher stress response on the east coast of New Zealand, where water nutrients are lower, than the west coast where nutrients are higher.

In Chapter Five, how mussels from one population respond to conditions at different sites with different environmental characteristics is tested.
Chapter Five hypothesis:

- Mussels from one source population transplanted to different sites with different environmental conditions will show different levels of stress response.

Chapter Six provides an overview and synthesis of the dynamics of stress response in mussels, with comparisons to the literature.

Chapter Two:

Development of molecular methods for use in mussel stress response studies

2.1 Introduction

The purpose of this chapter is to describe the development of molecular techniques for use in mussels. Stress response gene discovery for *Mytilus galloprovincialis* and *Perna canaliculus* was done and primers and quantitative polymerase chain reaction (qPCR) were developed and optimised. The MIQE guidelines for qPCR were followed closely to ensure necessary controls were utilised at each stage of the work (Bustin *et al.*, 2009).

2.1.1 Gene choice

The choice of genes used as a marker for physiological stress responses was guided mainly by the results of mussel microarrays done by Place *et al.* (2008) and Lockwood *et al.* (2010). Although Place *et al.* (2008) and Lockwood *et al.* (2010) used microarrays for mussels, the physical and technical resources required to enable a microarray were not available at the University of Canterbury. Orginally, it was anticipated that microarray chips were going to be available for me to use in collaboration with the Hofmann laboratory at the University of California Santa Barbara. However, due to changes in personel in the Hofmann Laboratory, access to the mussel microarray chips and the expertise for carrying out the experiments were no longer availabe to me at the time of this study. Due to the unavailability of microarrays, it was decided to pursue this research using RT-qPCR. Three laboratories with RT-qPCR facilities were contacted in an effort to establish a relationship that would allow the qPCR to be done. The three laboratories were: the Kennedy Laboratory at University of Otago School of Medicine in Christchurch, the Environmental Science and Research laboratories also in Christchurch and the Jameson laboratory the School of Biological Sciences at the University of Canterbury. The research was done at the University of Canterbury.

In the Place *et al.* (2008) study, gene expression patterns were tested from mussels living at four different sites in the Pacific northeast. Strawberry Hill, a site on the west coast of Oregon, is known to be more thermally challenging than some other sites on the west coast due to the timing of summer-time low tides (Helmuth *et al.*, 2002). The expression patterns of several genes differed between the more and less stressful field sites sampled. A subset of genes whose expression was different at Strawberry Hill in the Place *et al.* (2008) study were used as targets in the present study (*hsp70, elf2* and *tis11d*). In the Lockwood *et al.* (2010) study, the

effects of acute thermal stress on native (*M. trossulus*) and invasive (*M. galloprovincialis*) mussel gene expression was assessed using a microarray. Stress response genes (*hsp24* and *hsp90*) that were differentially expressed in the native versus invasive species were used in this study. Based on the microarray results from Place *et al.* (2008) and Lockwood *et al.* (2010), several other genes that responded to environmental stress were identified and tested for use in this study but they could not be sufficiently optimised (detailed in **Section 2.2.8** and **Table 2.2**) for use in reverse transcription quantitative PCR (RT-qPCR).

2.1.2 Sample tissue

Molecular studies in mussels typically use gill, mantle or muscle tissue. Gill tissue was used for this study because (1) it has been shown in *M. californianus* that stress-inducible gene expression is similar in gill, mantle and adductor muscle (Gracey *et al.*, 2008), (2) it is easily removed from the animal, (3) there have been several recent studies that use gill tissue to assess gene expression (Place *et al.*, 2008; Tomanek, 2010; Place *et al.*, 2012), (4) expression of proteins involved in stress response have been shown to be upregulated in the gills of *M. trossulus* (Hofmann and Somero, 1995) and (5) gill tissue responds robustly to physiological perturbations (Buckley *et al.*, 2001; Venier *et al.*, 2006; Place *et al.*, 2008).

2.1.3 Genomic DNA versus complementary DNA

For gene identification and PCR primer development, genomic DNA (gDNA) was used instead of complementary DNA (cDNA). cDNA is made by the reverse transcription of processed messenger RNA (mRNA) (Karp, 2004). mRNA is present when the cell is actively transcribing genes to make proteins. Only mRNAs present when the sample was collected will be found in a cDNA sample. This is ideal for qPCR, where the goal is to quantify amount of mRNA transcript, but gene identification and primer optimisation is best when all the genes are present and the presence of signal is not dependent upon active mRNA transcription. gDNA is simply all the DNA in the cell (Karp, 2004), making it ideal for gene discovery work.

2.1.4 RNA extraction

High RNA quality is essential for robust RT–qPCR (Bustin and Nolan, 2009). To do RT–qPCR, cDNA is reverse transcribed from RNA extracted from a

sample. The utmost care must be taken during RNA extraction to prevent degradation by ribonucleases (RNases) and to ensure the sample is free of inhibitors of the reverse transcriptase (Bustin and Nolan, 2009). Inhibitors may include remnants of reagents from the RNA purification or other components of the animal such as gut contents if a dissection was not carefully performed. RNases, RNA degradative enzymes, are present in most environments, including on human skin, and readily contribute to RNA degradation. cDNA is more stable than RNA and so it is generally used for qPCR (Bustin, 2004).

2.1.5 Primer design

Correct primer design is critical to achieving success in qPCR studies. When designing primers, a small amplicon is ideal (Bustin, 2004). Small products tend to amplify with higher efficiency than larger products and they are more likely to fully denature in the 92-95˚C step of the PCR reaction. Fully denatured products are more likely to be amplified in subsequent reactions. Small amplicons can be more quickly synthesised, which can prevent any contaminating gDNA from being amplified. gDNA tends to be much larger than cDNA due to the presence of introns and requires longer extension times. Short extension times for small amplicons help to prevent gDNA amplification (Bustin, 2004). In addition to a small amplicon size, primer design that spans an intron-exon border is also ideal. gDNA has not been spliced and so still has the introns in the sequence. When a primer pair spans an intron/exon boundary, a gDNA product will be large, since it would contain the intron, while a cDNA product would be small as it would not contain the intron (**Figure 2.1**). If the intron and exon boundaries are unknown, additional care must be taken to ensure that the sample is not contaminated by gDNA, such as the use of DNases, –RT controls and qPCR validation (Bustin *et al.*, 2009).

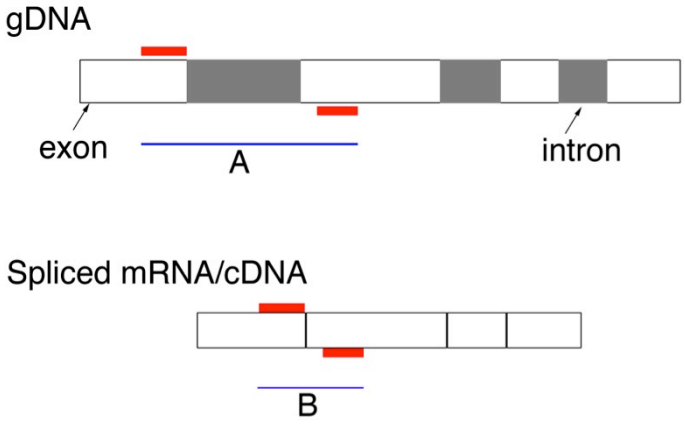


Figure 2.1 Schematic diagram of gDNA intron splicing. gDNA contains introns (grey sections) that are spliced out of the sequence during mRNA processing. PCR primers (red bars) that span an intron/exon boundary will result in larger or shorter products depending on if gDNA or cDNA is amplified (blue bars A and B respectively).

2.1.6 Data analysis

qPCR can be used for absolute or relative quantification. The most common method is relative expression analysis because the relative quantities are easier to measure and often more meaningful than absolute. The relative state is often more informative to the researcher by providing information about a treated sample compared to the control sample. Absolute quantification is often not necessary. There are multiple data analysis options available for relative qPCR studies. The ΔΔCt method (Livak and Schmittgen, 2001) is often used to calculate the relative expression ratio. However, that method requires a major assumption to be made. It assumes that the reaction has 100% efficiency, such that the product doubles at every cycle of the reaction. Perfect efficiency is rarely the case. This method is called ΔΔCt because it compares the change in expression of the control and the change in expression of the treated samples relative to the endogenous reference sample. When efficiencies of the reference gene and the target gene are not equal, the direct comparison between them can not be accurate as they amplify at different rates in the qPCR reaction (VanGuilder *et al.*, 2008).

The Pfaffl method of DNA analysis incorporates the reaction efficiencies into the relative expression calculations (Pfaffl, 2001). Modifications have been made to the Pfaffl formula allowing multiple reference genes to be included in the

calculations (Hellemans *et al.*, 2007). Stably expressed reference genes are critical to standardise between samples to ensure that the gene expression differences result from the treatment conditions rather than technical variability in RNA extraction, cDNA synthesis or loading error (Bustin *et al.*, 2009). Efficiency corrected calculation models that utilise multiple reference genes are ideal (Hellemans *et al.*, 2007; Pfaffl *et al.*, 2009).

2.1.7 Summary

The methods used for gene identification, sample collection, RNA extraction and cDNA synthesis are described in detail below. Also discussed is the optimisation of PCR primers, RT–qPCR quality controls (primer efficiency, standard curves and no template controls) and data analysis. The RT–qPCR done in the experiments for this thesis adhered strictly to the MIQE Guidelines (Bustin *et al.*, 2009).

2.2 Methods & Discussion

2.2.1 Sampling

For molecular analyses, gills were dissected from individual mussels in the field and placed into a 1.5 ml tube (**Figure 2.2**). The tubes were dropped into liquid nitrogen to flash freeze the tissue, then brought back to the laboratory and stored at −80˚C until ready to be processed.

Figure 2.2 A mussel (*M. galloprovincialis*) being dissected in the field. In this photo, the gills are cream-coloured and visible, flush with the valve on the right. The scalpel is pointing at the foot and byssal threads. The byssal threads were cut to remove the mussel from the rock.

2.2.2 Genomic DNA extraction (CTAB)

For gene identification work, genomic DNA (gDNA) was extracted from the gill tissue of control animals for use in polymerase chain reaction (PCR) (Weising *et al.*, 2005). Tissue frozen at −80˚C was crushed in liquid nitrogen with a plastic pestle. CTAB buffer (2% v/v; 600 µl) was added to the crushed tissue and allowed to incubate for 2 h at 60˚C. Following the incubation, the sample was spun for 2 min at 8000 x g and the supernatant was added to a new tube. Chloroform (700µl) was added and the sample was shaken vigorously followed by a 2 min incubation at room temperature. After another 2 min spin at 8000 x g, the upper aqueous phase was transferred to a new tube and 700 µl of isopropanol was added. Following 15 min at room temperature, the DNA filament was removed with a scored pipette tip and transferred to a new tube. The filament was washed three times with 80% (v/v) ethanol. After the wash, the sample was air dried for 10 min and allowed to dissolve overnight at 4˚C in 50 µl TE pH 8. The following day, the quantity of the sample was assessed with a NanoDrop® (Nyxor, ND-100) to determine the DNA concentration.

2.2.3 RNA extraction

RNA was extracted from the gill tissue of one to three mussels using the RNeasy Mini Kit (Qiagen) (Connor and Gracey, 2011). A total of 30 mg of tissue was used for RNA extraction for each treatment. Buffer RLT (600 µl) with ß-mercaptoethanol was added to the 30 mg of tissue, which was homogenised in a Bead Ruptor (Omni International) at 2.5 x g for 8 s using three 2 mm stainless steel ball bearings. The lysate was spun at 8000 x g for 3 min at room temperature. It was difficult to separate the supernatant from pellets while the stainless steel beads were still in the tube; therefore, the lysate was removed and transferred to a new tube, which was then spun at 8000 x g for an additional 3 min. The supernatant was then removed and transferred to a new tube. One volume of 70% (v/v) ethanol was added to the cleared lysate and gently mixed. In all of the following treatments, the flow-through was discarded. The sample was transferred to the RNeasy column (RNeasy Mini Kit, Qiagen) 700µl at a time and centrifuged at 8000 x g for 15 s. The lysate and 70% (v/v) ethanol mixture was added to the column and spun again for 15 s at 8000 x g. Buffer RW1 (350 µl;

RNeasy Mini Kit, Qiagen) was added to the spin column to wash the membrane and the column was then spun for 15 s at 8000 x g.

The samples were DNase-treated with the RNase-Free DNase Set (Qiagen) to prevent possible genomic DNA contamination. Lyophilised DNase was dissolved in 550 μl of RNase-free water to create a stock solution. Each sample received a Buffer RW1 (RNeasy Mini Kit, Qiagen) wash. Samples were then incubated at room temperature for 15 min with a mixture of 10 μl of DNase I stock solution and 70 μl Buffer RDD from the RNase-Free DNase Set (Qiagen). This was followed by another wash with 350 μl Buffer RW1 (RNeasy Mini Kit, Qiagen) and spun at 8000 x g for 15 s. The column was washed twice with 500 μl of Buffer RPE (RNeasy Mini Kit, Qiagen) and then spun at 8000 x g for 15 s. It was then placed in a new collection tube and spun at 8000 x g for 1 min to remove any residual traces of Buffer RPE, after which it was placed in a new 1.5 ml tube and the RNA eluted with 50 μl of RNase-free water by spinning at 8000 x g for 1 min. The quantity and quality of the RNA was assessed with a NanoDrop® (Nyxor, ND-100) and RNA integrity was checked by visualisation on a 1.5% (w/v) agarose gel. If the 260/280 ratio on the NanoDrop was below 1.90, or the gel was too smeared, the sample was considered degraded and was discarded and remade (**Figure 2.3**).

All agarose gels were done using the buffer TAE at pH 8.0. The ladder used to estimate product size and quantity was Hyper Ladder I (Bioline), the voltage was between 50-120 v. The gel was allowed to run from 20-50 minutes, until the products (visible with the loading dye) were approximately two-thirds of the way down the gel.

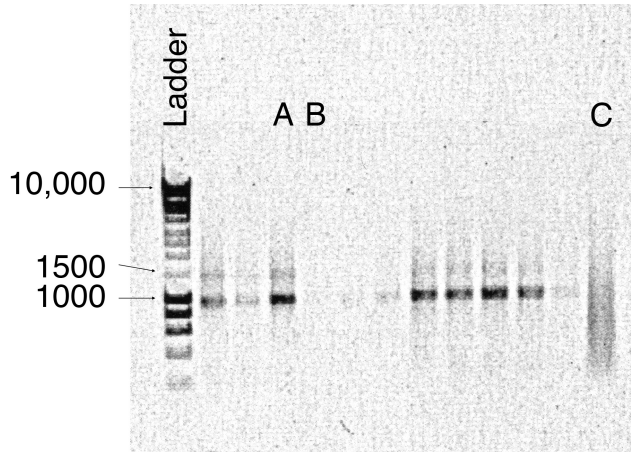


Figure 2.3 Agarose gel image of RNA extractions from mussel gills. Usable (A), failed (B) and degraded (C) samples are identified by the nature of the RNA band. Smeared bands result from sample degradation, sharp bands indicate samples with intact RNA. The absence of a band indicates that RNA extraction failed.

2.2.4 cDNA synthesis

Total RNA (500 ng) was reverse-transcribed into complementary DNA (cDNA) using 100 pmol of random primers and 50 units of Expand Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany). The reverse transcription (RT) reaction was incubated for 4 h at 42˚C. cDNA was diluted 10 fold with Nano Pure water and stored in the −20˚C freezer. Every cDNA synthesis reaction was run in duplicate. One reaction (+RT) included the reverse transcriptase enzyme and one reaction (–RT) omitted the enzyme. The –RT reaction is used to ensure there was no gDNA contamination. Any amplification in a qPCR reaction that occurred in the –RT sample would be a result of gDNA contamination. qPCR is used to quantify the amount of messenger RNA (mRNA) gene transcripts in a sample, gDNA contamination could falsely enhance the amplification curve and result in incorrect results. As a measure of quality control, for potential gDNA or other contamination, qPCR reactions of +RT and –RT samples were run with the reference genes *18S* and *actin* to check the quality of the cDNA. –RT reactions should have no amplification, and all the good cDNA samples should have similar amplification properties (**Figure 2.4**). Any samples with abnormal amplification plots or melt curves were discarded and remade.

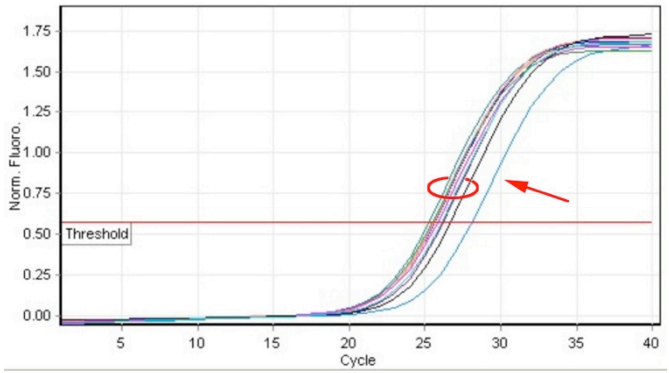


Figure 2.4 *Actin* amplification plot of mussel gill RT–qPCR. Good (circle) and abnormal (arrow) cDNA samples. RT–qPCR was performed on gill tissue. A non-contaminated –RT sample would have a flat amplification curve at 0.00 Normalised Fluorescence.

2.2.5 PCR primer design

PCR primers were designed with either Primer Premier™ 5.0 or Primer3 (Rozen and Skaletsky, 2000). Primer Premier™ 5.0 provided information about predicted hairpin structures, primer dimers and false priming. Additionally, with Primer Premier™, the position of the primer can be set and the suitability of that location assessed. Primer3, on the other hand, returns ten possible primers but the location of these primers can not be manipulated easily base by base in the web interface. Primer length was generally between 18 and 25 oligonucleotides with a melting temperature of around 60˚C.

2.2.6 PCR

PCR reactions were performed with the following reagents: 2.5 μl 2 mM dNTPs, 2 μl 10 × Taq Buffer (Roche), 1 to 2 μl 25 mM $MgCl_2$ (adjusted to optimise reaction), 10 pmol primers (sense and anti-sense), 2 μl DNA template, 1 unit Taq polymerase (Roche), water to 20 μl. The standard PCR program was 35 cycles of: 94˚C for 40 s, 50-60˚C for 30 s, 72˚C for 30 s, followed by 5 min at 72˚C, then held at 4˚C.

2.2.7 Primer validation

PCR amplification for gene identification was done with genomic DNA from control animals collected under natural conditions. After primers were designed,

PCR was tested at varying annealing temperatures about 10˚C either side of the predicted primer annealing temperature to identify the optimal PCR reaction annealing temperature. PCR products were separated on a SYBR Safe™-stained 1% (w/v) agarose gel, run at 120 V for 30 min. The reaction with the strongest bands on an agarose gel was sequenced to confirm the identity of the amplified product (**Figure 2.5**). Bands of the expected size were cut out under blue light from a Chemi Genius2 BioImaging System (Syngene) and purified using an Agarose Gel DNA Extraction Kit (Roche). The amount of PCR product was quantified using Hyperladder I (Bioline) and a NanoDrop®. The purified PCR product was sent to Macrogen Inc, South Korea (www.macrogen.com) for sequencing. Independent sequencing of the forward and reverse strands was obtained with the same primers that were used in the PCR amplification reaction. The sequences were then used to BLAST search the NCBI database to verify the product as the gene of interest.

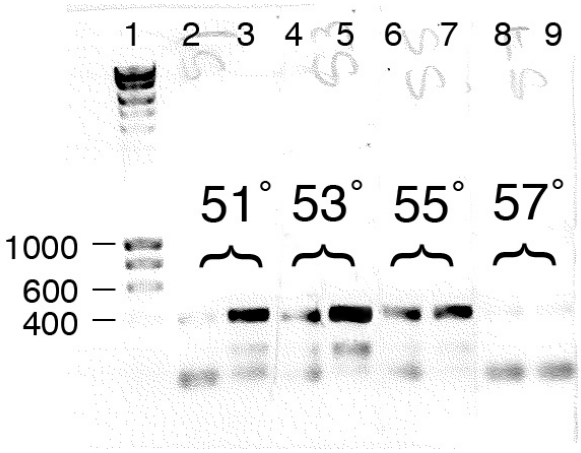


Figure 2.5 Agarose gel image of temperature gradient PCR products of mussel gill tissue for the gene *elf2*. This PCR tested annealing temperatures (51-57˚C) in two different template samples from *M. galloprovincialis*. Sample one is in lanes 2, 4, 6 and 8, sample two is in lanes 3, 5, 7 and 9. Sample one amplified poorly. For this primer pair, the optimal annealing temperature was 53˚C, the product size is 371bp. Size markings (base pairs) on the DNA ladder are visible on the left.

2.2.8 Gene choice

Reverse transcription quantitative polymerase chain reaction (RT–qPCR) was performed to determine the expression of five known stress response genes (Place *et al.*, 2008; Lockwood *et al.*, 2010). The expression levels of three heat shock

proteins (*hsp24, hsp70* and *hsp90*), a transcriptional regulator (*elf2*) and a cell cycle regulator (*tis11d*) were measured in *M. galloprovincialis*. The expression level of only one heat shock protein (*hsp70*) was measured in *P. canaliculus* due to limited genomic sequence availability and low sequence conservation between *Perna* and *Mytilus*. For example, primers worked well on DNA from three different species of mussels in the genus *Mytilus (M. galloprovincialis, M. trossulus,* and *M. californianus*) and yet failed to amplify anything in reactions with DNA from *P. canaliculus* (**Figure 2.6**). Extensive work was done in an effort to isolate additional *P. canaliculus* genes (detailed below), but in the end only *hsp70* could be successfully amplified for RT–qPCR. Primer sequences are shown in **Table 2.1**. When products were < 200 base pairs (bp), they were sub-cloned into a bacterial plasmid (**Section 2.2.9**) prior to sequencing.

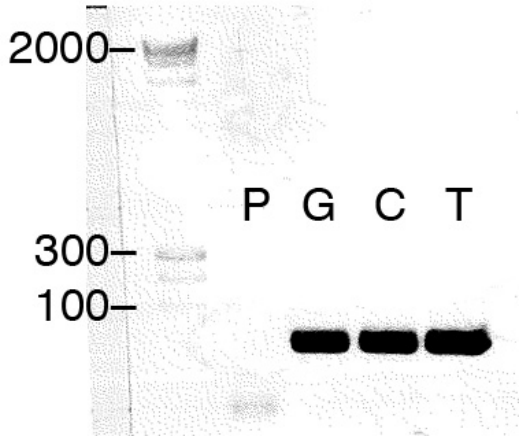


Figure 2.6 Agarose gel image of *elf-a* PCR products for the gill tissue from four different species of mussel. P: *P. canaliculus;* G*: M. galloprovincialis;* C*: M. californianus;* T*: M. trossulus*. No amplification occurred in the *P. canaliculus* sample. Size markings (base pairs) on the DNA ladder are visible on the left, product size is 66 bp.

Sequences of target genes are provided in **Appendix A** and are aligned with the top hit from the NCBI Basic Local Alignment Search Tool (BLAST) to show identification**.**

Table 2.1 Sense (s) and anti-sense (as) sequence of primer pairs used in RT–qPCR. Product length (base pairs: bp). The GenBank or Mytibase accession number is reported.

Gene	Sequence	Product length (bp)	Accession number
	Reference genes for both species		
18S (s)[2]	5'-TCGATGGTACGTGATATGCC-3'	84	L33452[1]
18S (as)[2]	5'-CGTTTCTCATGCTCCCTCTC-3'		
28S (s)	5'-GGTGTAGCATAGGTGGGAGC-3'	563	AB103129.1[1]
28S (as)	5'-AACGACTTGTCATCAGTAGGGT-3'		
actin (s)[2]	5'-CTCTTGATTTCGAGCAGGAAA-3'	138	AF157491[1]
actin (as)[2]	5'-AGGATGGTTGGAATAATGATT-3'		
	***M. galloprovincialis* target genes**		
hsp24 (s)	5'-TTGGAACCGCTACAATCAGTC-3'	102	JF803805.1[1]; MGC00301[4]
hsp24 (as)	5'-TGACGACCATACCCATAACCTAC-3'		
hsp70 (s)[3]	5'- CTTGTTGGTGATGCAGCTAAAAA -3'	64	AJ585375.1[1]
hsp70 (as)[3]	5'-TTGGCATCGAAGATTGTATTTGA-3'		
hsp 90 ex2 (s)	5'-TTGCCAAGTCTGGTACTAAAGC-3'	107	AJ586906.3[1]
hsp 90 ex2 (as)	5'-AGCAACCAGGTAGGCGGAGTAG-3'		
elf2 (s)	5'-CCGCAAGATGACGATGAATGAC-3'	371	MGC04450[4]
elf2 (as)	5'-TAGGTGGATTATCAACTCCTTTGTAG-3'		
tis11d (s)	5'-ACAGAATCGCAGAAACCAGC-3'	431	MGC01771[4]
tis11d (as)	5'-AGACCAGCAGAAGCAACACT-3'		
	***P. canaliculus* target gene**		
hsp70 P (s)	5'-TTGAGTTGACAGGAATCCCACC-3'	164	MGC01310[4]
hsp70 P (as)	5'-TTCTTTGCTTAGTCGTCCTTTGTC-3'		

(1 NCBI; 2 Dondero *et al.*, 2005; 3 Place *et al.*, 2008; 4 Venier *et al.*, 2009)

In an effort to isolate additional genes for expression analyses, many techniques were used. Multiple sets of primer pairs for each gene were designed and tested in *M. galloprovincialis* and *P. canaliculus* samples (**Table 2.2**). Some of these primer pairs could be mixed and matched to enable possible amplification of different sized products.

Degenerate primers were designed in an effort to amplify target genes. The degenerate primers were designed by aligning the DNA or protein sequences from several different species of molluscs and other organisms (e.g., *Crassostrea* sp. (oyster) *Dreissen polymorpha* (zebra mussel), *Haliotis tuberculata* (abalone), *M. galloprovincialis* (mussel), *Ostrea edulis* (oyster), *Elysia timida* (nudibranch), *Xenopus leavis* (frog), and *Carassius auratus* (fish)). The multiple alignment was then used to identify conserved regions in the sequence. Primers were designed with degeneracy to account for sequence differences between species in an effort to amplify a product from *P. canaliculus* DNA samples without having the sequence directly.

Two sets of *P. canaliculus* primers were received from Dr. Andrew Fiddler at the Cawthron Institute for varying isoforms of *hsp70*. The primers did amplify DNA products in this study but there were other primer pairs that I had already designed that were successful for this gene.

When primer pairs were being tested, the annealing temperature in the PCR reaction and the magnesium concentrations were varied from 1 mM to 4 mM in an attempt to amplify DNA.

During primer optimisation, positive and negative control reactions were always included. Therefore, it was evident if the reaction itself failed or if the new primer pairs were not yet working when reactions were unsuccessful. Considerable time was spent in an effort to amplify additional genes in *P. canaliculus*. However, only one target gene could be used to advance the experiments and allow time to run the analyses on mussel gene expression due to the limited time restrictions for the PhD degree

Table 2.2 Sense (S) and anti-sense (AS) sequences of primer pairs that were unsuccessful for gene identification.

Gene	Primer	Sequence	Reference
elf2	S2	5'-AGCTTATGGATGGAAAGAGGTGTAG-3'	
elf2	S3	5'-CCAAAATCTGGCAAATTCCCGAAG-3'	
elf2	S4	5'-AGATATTACTACAAGCGAGGCATTC-3'	
elf2	S5	5'-GTCTGCTGAAATGGGAAGATAAAG-3'	
elf2	AS2	5'-TTTCTTCGGGAATTTGCCAG-3'	
elf2	AS3	5'-GGAATTGGAGCCAAACTTGTAC-3'	
elf2	AS4	5'-TCAGTTTCTCGTAGGTCATCCC-3'	
elf-α	S1	5'-GCATCTGGTACTGGTGAGTTTGAA-3'	(Place *et al.*, 2008)
elf-α	AS1	5'-AGGGCGTGTTCTCTTGTCTGA-3'	(Place *et al.*, 2008)
elf-α	S2	5'-TCCCTGAGGGTATTAAGGGTG-3'	
elf-α	AS2	5'-TGGTGGTTCAGTGTTGTCCA-3'	
elf-α	S3	5'-TGGCTGGCACGGAGACAAC-3'	
elf-α	S4	5'-ATGTTACCATTATTGATGCTCCTGG-3'	
elf-α	AS3	5'-TCTTTCCGCTGGCATTGC-3'	
elf-α	AS4	5'-AGAGATACCAGCTTCAAATTCACC-3'	
hsc71	S	5'-TGCCTTCACAGACACCGAAA-3'	(Place *et al.*, 2008)
hsc71	AS	5'-GACTGGGTTCATTGCCACTTG-3'	(Place *et al.*, 2008)
hsc71	S2	5'-AACAACCCCAAGCTATGTCG-3'	
hsc71	S3	5'-GGGCAATGGATAGGAAGTCA-3'	
hsc71	AS2	5'-CGGACACAAGATCTAGAAGAGAAA-3'	
hsc71	AS3	5'-AGATACACTACCAAAGTCGGGA-3'	

Table 2.2 (Continued) Sense (S) and anti-sense (AS) sequences of primer pairs that were unsuccessful for gene identification.

Gene	Primer	Sequence	Reference
hsp70	S1	5'-TGATGCCAATGGTATCCTGAATG-3'	
hsp70	S2	5'-TGCCAATGGTATCCTGAATGTATC-3'	
hsp70	AS2	5'-TGTTTCTCGTCTTCTGCCTTG-3'	
hsp70	AS1	5'-TTCTCGTCTTCTGCCTTGTATTTC-3'	
hsp70	S3	5'-AGGAAAGGGTCCAGCAATCG-3'	
hsp70	AS3	5'-TTGTAGCGTTCAATGCGACTTG-3'	
hsp90 ex1	S1	5'-CTGTCTTTTTAAGCGTGGTCAAGC-3'	
hsp90 ex1	S2	5'-ACTGGTGACCTCCCCTTGTTG-3'	
hsp90 ex8	AS1	5'-GTGGCTCAGTGTGGTGTTCCTC-3'	
hsp90 ex8	AS2	5'-GCATGACTCTGTGGTTCCTCC-3'	
psmb1	S1	5'-GAATGGAGGGACAGTATTAGCAGTT-3'	
psmb1	S2	5'-GAATGGAGGGACAGTATTAGCAGTT-3'	
psmb1	S3	5'-GAATGGAGGGACAGTATTAGCAGTT-3'	
psmb1	S4	5'-GCCATGTTGTCTACTATGCTTTACTCTAG-3'	
psmb1	S5	5'-AGGAGTCACATTATGCTCCTAAACAG-3'	
psmb1	AS1	5'-AGCATCACCAGTGTATATGTCTCTTCC-3'	
psmb1	AS2	5'-GAAAGAGACATATACACTGGTGATGCT-3'	
psmb1	AS3	5'-AGCATCACCAGTGTATATGTCTCTTTC-3'	
psmb1	AS4	5'-GCTCTGTAGGTTTCCCTTTCATAAG-3'	
psmb1	AS5	5'-AGGTCTTTGGTAGATCCCTTGTGTG-3'	

Table 2.2 (Continued) Sense (S) and anti-sense (AS) sequences of primer pairs that were unsuccessful for gene identification.

Gene	Primer	Sequence	Reference
psmb1 PC	S6	5'-TTGCTGGACTAGATGATGAAGG-3'	
psmb1 PC	S7	5'-GTGATGGTGCGGTGATACGG-3'	
psmb1 PC	AS6	5'-GGACGTAGGACGTTAGGGTG-3'	
psmb1 PC	AS7	5'-TCCCTTGTATGAATGGAGAAACC-3'	
tis11d	S2	5'-CGGTGACCGAGCTTTGAGCAGT-3'	
tis11d	S3	5'-TGCCGACCCTTCGAGGAAAGTGG-3'	
tis11d	S4	5'-GAGAATTTACATCGCAAGTTGGATAGGAG-3'	
tis11d	S5	5'-ATCGCAAGTTGGATAGG-3'	
tis11d	S6	5'-TGCCGACCCTTCGAGGAAAGTGG-3'	
tis11d	S7	5'-CGGTGACCGAGCTTTGAGCAGT-3'	
tis11d	AS2	5'-TGCGCTAGCATGGCTTCGTGATTC-3'	
tis11d	AS3	5'-CACCGTGAGCAAACTGACACTTATC-3'	
tis11d	AS4	5'-CACCGTGAGCAAACTGACACTTATC-3'	
tis11d	AS5	5'-CAGTAAAGCAAGTGTTGC-3'	
tis11d	AS6	5'-CACCGTGAGCAAACTGACACTTATC-3'	
tis11d	AS7	5'-TGCGCTAGCATGGCTTCGTGATTC-3'	
mxi1	S1	5'-AGCACCAGCTGGAGGTTCTA-3'	
mxi1	AS1	5'-GTCAGCGCCAGTATCAGTGA-3'	

Table 2.2 (Continued) Sense (S) and anti-sense (AS) sequences of primer pairs that were unsuccessful for gene identification.

Gene	Primer	Sequence	Reference
Primers received from Andrew Fiddler of the Cawthron Institute			
AFHSP70For	S	5′- TAYTCCTGTGTTGGAGTNTTYCARCA -3′	A. Fiddler, Cawthron
AFHSP70Rev	AS	5′-NGCICCRTAIGCIACAGCYTCATCNGGRTT -3′	A. Fiddler, Cawthron
HsGp-I-For1	S	5′- CTNACIATAGACGAGGGITCYWTITTYGA -3′	A. Fiddler, Cawthron
HsGp-2-For1	S	5′- TCIATTCTGACNATIGAGGAYGGIATYTTTGA -3′	A. Fiddler, Cawthron
HsGp-12-Rev1	AS	5′- ITTITTGTCYTTIGTCATIGCICIYTCNCC -3′	A. Fiddler, Cawthron
Degenerate Primer Pairs			
elf-α	S1	5'-GGCTCCTTCAAGTATGCCTGG-3'	
elf-α	AS1	5'-CTGGCACWGTTCCAATACCTCC-3'	
psmb1	S1	5'-AGGGACWGTRYTWGCWGTTGCYGG-3'	
psmb1	AS1	5'-AAMACASMTCCTTTACCYTCMTCATC-3'	
tis11d	S1	5'-WCCCTTYGARGAAAGTGGMC-3'	
tis11d	AS1	5'-GGWCCRTASGGACARAAWCC-3'	

2.2.9 Sub-cloning

Small PCR products (< 200 bp) were ligated into pGem-T Easy, a vector using T4 DNA ligase, 1 μl pGEM®-T Easy Vector System I following the manufacturer's instructions (Promega, 2010) (Varsani *et al.*, 2008). The vector with the insert was then transformed into chemically competent *E. coli* (DH5α) and grown on X-gal (40 ug/ml), IPTG (1 mM) and ampicillin (100 ug/ml) LB agar plates. Using blue/white selection (colonies with the vector but no insert were blue, and bacterial colonies with no vector were unable to grow because they lacked ampicillin resistance), white colonies were picked and grown in Luria + ampicillin broth at 37˚C overnight. The plasmids were then isolated from the *E. coli* with an ISOLATE Plasmid DNA Mini Kit (Bioline) as per the manufacturer's instructions. The isolated plasmid vectors were then sent to Macrogen Inc, South Korea (www.macrogen.com) for sequencing using universal M13 primers. To confirm the identity of the PCR products, the NCBI Basic Local Alignment Search Tool (BLAST) was used to search for sequences that matched the query.

2.2.10 Reference gene identification

RT–qPCR requires stably expressed reference genes (Bustin *et al.*, 2009). The stability of reference gene expression can be ascertained through a variety of computational methods (GeNorm: Vandesompele *et al.*, 2002; Normfinder: Andersen *et al.*, 2004; BestKeeper: Pfaffl *et al.*, 2004; ΔCt: Silver *et al.*, 2006). A web-based tool, RefFinder, has been developed that integrates the four major mathematical models into one easy-to-use tool (Xie, 2012). In RefFinder, the candidate reference gene data is analysed with each program (GeNorm, Normfinder, BestKeeper and ΔCt) and based on the rankings from each individual assessment, a final rank is assigned to each candidate reference gene (**Figure 2.7**) (Xie, 2012). Based on the results from RefFinder, *18S* is the most stable reference gene tested and *elfa* is the least stable. Further discussion about the final choice of reference genes for these studies is below.

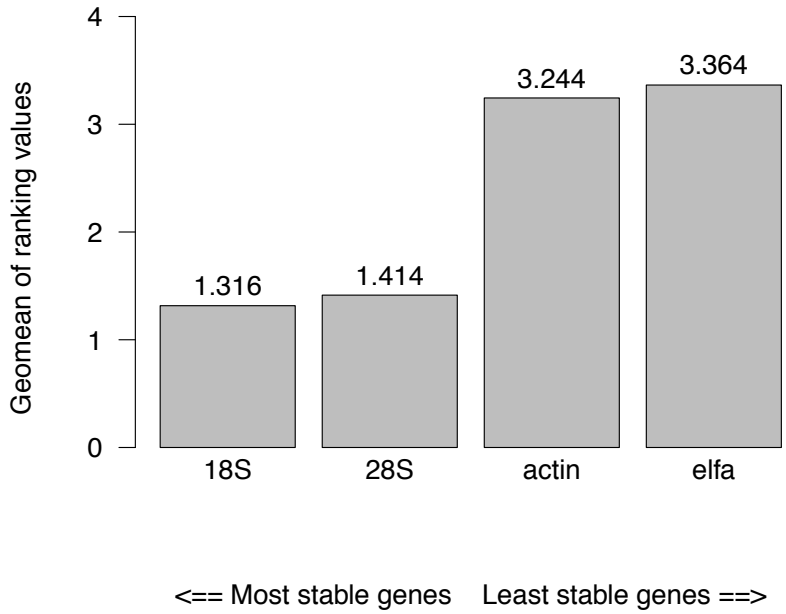


Figure 2.7 Comprehensive rank for reference gene stability from RefFinder. Ranks are based on four different algorithms (Normfinder, BestKeeper, GeNorm, ΔΔCT) that assess the consistency of reference gene expression (Xie, 2012).

Another important feature in qPCR is the specificity of primer pairs. qPCR specificity can be verified through genetic sequencing of the products as well as through the melt curve characteristics. The melt curve takes place at the end of the qPCR reaction when the products are melted slowly while fluorescence is measured at every one degree temperature step. SYBR Green, a double stranded DNA binding compound emits light when bound by double stranded DNA (Bustin, 2004). When PCR products that were created in a reaction containing SYBR are melted into single-stranded pieces of DNA, the qPCR machine can measure the fluorescence emitted. Pure qPCR products will have a large change in fluorescence at a discrete temperature as the majority of the product will melt into single stranded DNA at a specific temperature. Two peaks, or wide peaks, suggest more than one product was created in the PCR reaction (Bustin, 2004) (**Figure 2.8**). Low temperature melt curves suggest primer dimer formation. Primer dimers result when the forward and reverse primers anneal to each other instead of the template DNA.

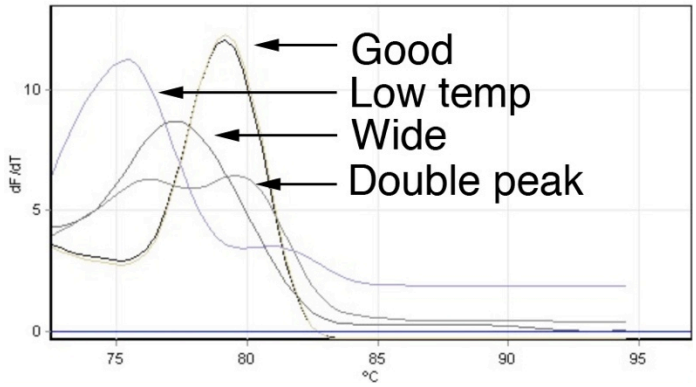


Figure 2.8 Examples of the possible variability in melt curves from *M. galloprovincialis* RT–qPCR reactions. Good melt curves are tall and narrow, indicating a pure product. Poor curves can have double peaks or a wide base indicating the melt of impure products. A low melting temperature is also an indication of a poor melt curve, implying that the melt may be the result of primer dimers having formed.

Four candidate reference genes were assessed for suitability: *18s, 28s, actin* and *elf*-α. *Elf*-α has been used as a reference gene in mussel stress response studies (Place *et al.*, 2008; Dutton and Hofmann, 2009). *Elf*-α was deemed unsuitable in the current studies for two reasons (1) it was the least stably expressed of the reference genes trialed (**Figure 2.7**), and (2) clean melt curves could not be achieved (**Figure 2.9I**). The three remaining reference genes were stably expressed in gill tissue of *M. galloprovincialis* and *P. canaliculus* (**Figure 2.7**) and the melt curves suggested that pure product was formed in the RT–qPCR reactions (**Figure 2.9A-C**).

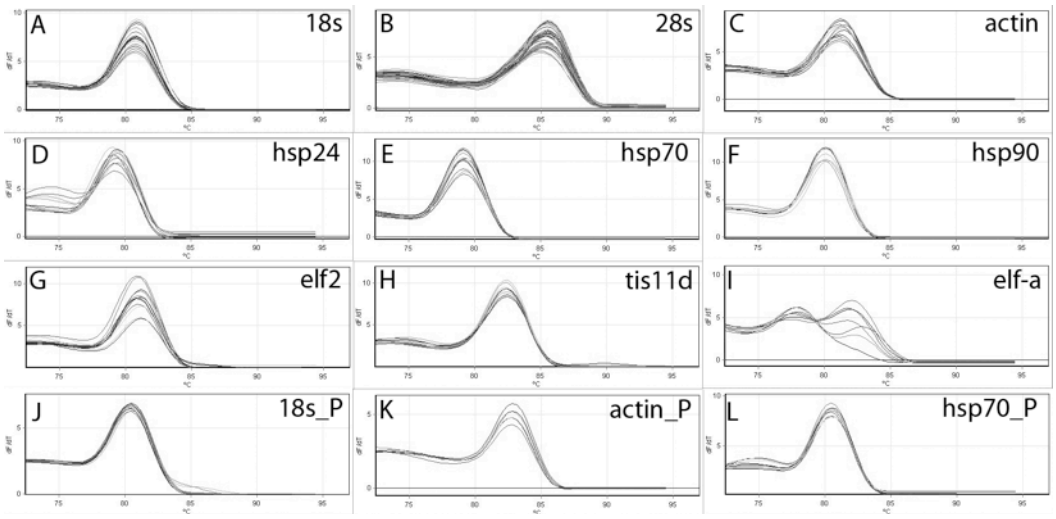


Figure 2.9 Representative RT–qPCR melt curves of *M. galloprovincialis* gill tissue for each primer pair used in these studies. Primer pairs are presented in **Table 2.1**.

2.2.11 Efficiency

Robust and precise qPCR analyses generally require high primer efficiencies (Bustin *et al.*, 2009). The amplification efficiencies of primer pairs depend on the sequence specificity of the primers and template, the thermal conditions in the reaction, purity of the template and the presence/absence of inhibitory factors in the reaction (Bustin, 2004). Serial dilutions of the template are used to create a calibration curve to determine the efficiency of primer pairs in qPCR (**Figure 2.10A**) (Bustin, 2004; Bustin *et al.*, 2009; Pabinger *et al.*, 2009). Calibration curves are a good measure of qPCR efficiency and the robustness of an assay because they are easily reproduced, rapid and provide information about the analytical sensitivity of the reaction (Bustin *et al.*, 2009). The efficiency is calculated as the slope of the log linear portion of the calibration curve (**Equation 2.1**). The slope used in the efficiency calculation is derived from a plot with the logarithm of the initial template concentration (the independent variable) plotted on the x-axis and the C_q (the dependent variable) plotted on the y-axis (**Figure 2.10B**). The theoretical maximum efficiency of 1.00 (or 100%) occurs when the amount of product doubles each cycle. Efficiency values of about 10% either side of 100% can be considered acceptable (Application Note Applied Biosystems, 2011). Efficiencies outside that range are sometimes acceptable if other factors, such as melt curve, and stability of expression, are deemed suitable (Bustin, 2004). In the present study, the assumption was made that the calculated efficiencies are assay

dependent but sample independent (Hellemans *et al.*, 2007; Pabinger *et al.*, 2009). As a result, efficiencies were generated using pooled cDNA samples from nine mussels that had been part of the experiments. However, the efficiency of each individual sample was not calculated. Standard curves from serial dilutions were built from triplicate RT-qPCR reactions. In the most common form of qPCR data analyses, ΔΔCt, the calculations are done assuming that the reactions are 100% efficient and that all primer pairs have the same efficiency (Livak and Schmittgen, 2001; Bustin, 2004; Hellemans *et al.*, 2007; Schmittgen and Livak, 2008; Bustin *et al.*, 2009; Pabinger *et al.*, 2009). However, efficiencies are rarely 100% and different primer pairs rarely have the same efficiency, which can cause substantial error in the results where gene expression levels are over or under estimated (Hellemans *et al.*, 2007; Pabinger *et al.*, 2009). Using the modified Pfaffl method (Pfaffl, 2001; Hellemans *et al.*, 2007), the efficiency values of multiple reference genes and the gene of interest are included in the calculation, which provides a better estimate of gene expression. The efficiencies for all the primer pairs used in this study are reported in **Table 2.3**.

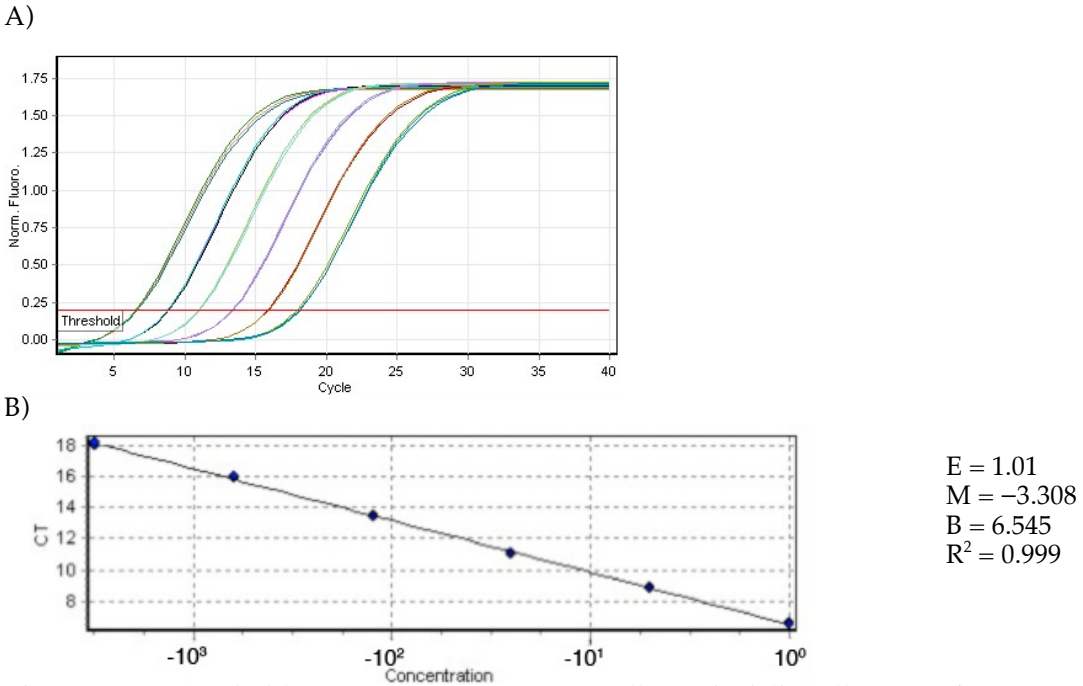


Figure 2.10 Serial dilution RT–qPCR in *M. galloprovincialis* gill tissue for 18S. A) Serial dilution amplification plot from the Rotor-Gene Q qPCR machine. The normalised fluorescence is on the y-axis and the reaction cycle number is on the x-axis. The horizontal red line is the threshold fluorescence level whose value is set by the Rotor-Gene Q software. B) Calibration curve. Y-axis has the quantification cycle number and the x-axis has the template concentration. E, efficiency; M, slope; B, Y-intercept.

$$E = 10^{\frac{-1}{slope}} - 1$$

Equation 2.1: qPCR efficiency. E is the efficiency and slope is derived from the calibration curve plot (**Figure 2.10**).

Table 2.3 PCR amplification efficiency of primer pairs.

Gene	slope	y-intercept	R^2	Efficiency
		M. galloprovincialis		
18s	−3.31	6.55	0.999	1.01
28S	−4.04	6.10	0.997	0.77
Actin	−3.23	23.99	0.995	1.04
hsp24	−3.23	22.65	0.997	1.04
hsp70	−3.40	15.71	0.997	0.97
hsp90	−3.16	15.34	0.996	1.07
elf2	−3.39	24.08	0.998	0.97
tis11d	−3.38	21.14	0.99	0.98
		P. canaliculus		
18s	−3.00	5.16	0.986	1.15
28s	−3.48	5.53	0.999	0.94
Actin	−3.15	29.94	0.962	1.08
hsp70	−3.10	14.59	0.989	1.10

2.2.12 RT–qPCR

Expression levels of the genes were normalised to three reference genes, *18S*, *28S* and *actin* in order to correct for RT reaction efficiency and possible RT–qPCR loading error. RT–qPCR reactions were performed in a 10 µl reaction with 1 µl of cDNA added to 5 µl SYBR Fast RT–qPCR kit 2X master mix (Kapa). 0.5 µl of each primer was added to the master mix. Primer concentration was validated, based on melt curves, high efficiency and low C_q values. Each reaction was run in triplicate on a Rotor-Gene Q (Qiagen) machine. After a 3 min melt at 95˚C, the 40 cycle reaction parameters were 95˚C for 10 s, 57˚C for 15 s, 72˚C for 20 s. The melt curve ran from 72-95˚C at 1˚C steps with a 5 s hold at each degree. The quality of the melt curve was assessed for every reaction (e.g., **Figure 2.8**) and the reaction discarded and repeated if it was impure. A plate standard was used on every RT–qPCR run and the C_q values were standardised across all plates to correct for inter-

run variability in the machine. No-template controls (NTCs) were used for each RT–qPCR run to ensure that qPCR reagents were not contaminated with template DNA. NTCs should have no template to amplify (**Figure 2.11**).

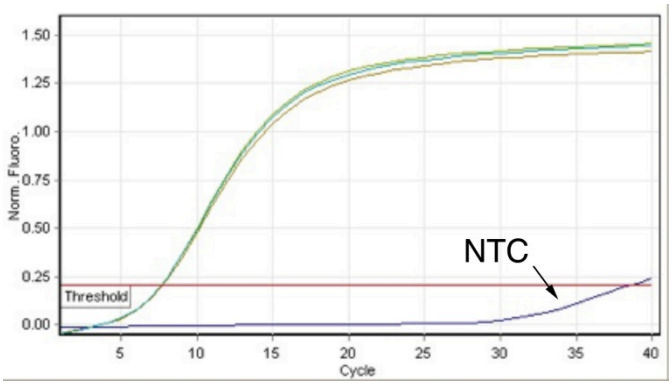


Figure 2.11 RT–qPCR amplification plot for representative *18S M. galloprovincialis* gill tissue samples. The no-template control (NTC) sample does not amplify due to the absence of template.

2.2.13 Data analysis

Data analysis was carried out using R statistical software (version 2.14.1) using a modified Pfaffl equation that utilises the geometric mean of the terms in the denominator (Pfaffl, 2001; Hellemans *et al.*, 2007) to accommodate three reference genes and their primer efficiencies (**Equation 2.2**).

$$R = \frac{(E_{target})^{\Delta C_q target(cont-treat)}}{\sqrt[3]{\{(E_{r1})^{\Delta C_q r1(cont-treat)}\} + \{(E_{r2})^{\Delta C_q r2(cont-treat)}\} + \{(E_{r3})^{\Delta C_q r3(cont-treat)}\}}}$$

Equation 2.2: Modified Pfaffl equation used to calculate expression of target genes relative to the reference genes. R, relative expression; E, Efficiency; C_q, quantification cycle; target, target gene; r, reference gene; cont, control sample; treat, treated sample (Pfaffl, 2001; Hellemans *et al.*, 2007).

2.2.14 Replication

The high cost of molecular biology reagents needs to be considered when designing ecological experiments that contain molecular components. Ecological studies often have elaborate experimental designs, much more so than molecular biology. As a result they can easily become overly complex for reasonably-priced

molecular analyses to be included. For RT-qPCR, in an ideal situation, three biological replicates would be collected, followed by three complete series of RNA extraction and cDNA synthesis. Each of the three biological replicates would then be run in triplicate for the qPCR. Many contemporary studies are published without adequate replication and representation of variability is frequently omitted (e.g., Dutton and Hofmann, 2009; Pantile and Webster, 2011; Núñez-Acuña *et al.*, 2012). While there were some problems with replication in my studies due to sample mortality in field experiments and cost-considerations of sample processing, biological and technical replication were maintained whenever possible. Often, three biological samples were pooled prior to tissue processing, which led to the variability among the three samples being encompassed in one replicate. As such, biological variability still existed in the data but it was not represented outright in the mathematical calculations of statistical significance.

Only three individuals were pooled due to sampling limitations. The experiments in Chapter Four were the first set of experiments done, and there were 24 plots at each of four sites. Mussel collections and dissection of tissue of approximately 30 individuals was the maximum number that could be processed in one tidal period. It was important to process the samples immediately to ensure that gene expression was not affected by the collection process. Furthermore, collection of all samples for one site on the same day was also important for maintaining the same conditions for all animals used in the study. Effectively the same method of pooling was used in subsequent studies (those in Chapters Three and Five) as for Chapter Four to preserve consistency across experiments.

2.3 Conclusion

The necessary controls were done at each step of the process to ensure high quality results at the final step of the process. As described throughout this chapter, these controls included ensuring high quality RNA, prevention of genomic DNA contamination, good quality cDNA, efficiency of primers, clean dissociation curves, inter-plate calibrators to standardise any between-run variability, negative control reactions in the cDNA synthesis, positive and negative control reactions in qPCR. The following three chapters use the RT–qPCR techniques described above.

Chapter Three:

Stress response reveals local patterns in mussel survival, growth and gene expression

3.1 Introduction

The study of physiological ecology attempts to understand the environmental factors that influence organisms and set their biogeographic distribution. Physiological ecology can be informative to aid in the prediction of the effects of climate change. In the intertidal zone, marine organisms are at risk due to changes in climate (Somero, 2011) and intertidal organisms must cope with both aerial and aquatic conditions. During aerial exposure, organisms can be subject to stressful parameters such as desiccation, elevated thermal conditions, and the inability to feed and excrete waste products (Somero, 2002). There are other conditions that can stress the organisms when the intertidal animals are submerged during high tide such as predation (e.g., fish and crab in Rilov and Schiel, 2006b; seastars in Pincebourde *et al.*, 2012) and sometimes extreme hydrodynamic forces (Menge, 1976). The diversity is extremely high despite the challenging conditions in this habitat. For example, it has been shown that hundreds of different taxa take refuge among mussels (Tsuchiya and Nishihira, 1986; Suchanek, 1992). High diversity in the intertidal zone suggests that the organisms are able to cope with the stressful conditions. The intertidal zone is an excellent system to study the physiological effects of environmental stress and climate change due to the naturally extreme conditions.

The vertical position on the shore occupied by a given species is set by a combination of environmental parameters and interspecific competition. The environmental conditions are more difficult high in the intertidal zone than lower down. Therefore, organisms with higher physiological tolerances often live at higher in the intertidal zone. Conditions are more favourable lower on the shore, where space is at a premium and only the competitively dominant species are able to persist. A well-known example of this zonation is the distribution of barnacles in the intertidal zone (Connell, 1961; Wethey, 1983). In Scotland, the low-zone species *Semibalanus balanoides* is dominant because it overgrows and crushes the mid-zone species *Chthamalus stellatus* (Connell, 1961). However, *S. balanoides* does not occur above a certain point due to physiological limitations, thus *C. stellatus* is free to occupy the mid-zone (Connell, 1961). Zonation patterns seen in the rocky intertidal have been studied extensively by marine ecologists. A lot can be understood about the interspecific species interactions and differences in

physiological tolerances between different species by understanding patterns of zonation.

Intertidal organisms often have plastic phenotypic responses to stress. For example, the threshold for induction temperature of chaperone proteins is elevated by several degrees in sea urchins (*Strongylocentrotus purpuratus*) adapted to warmer southern regions relative to urchins from more northerly (colder) areas (Osovitz and Hofmann, 2005). Physiological performance can be optimized over a range of conditions to enhance fitness and persistence at a given location. Plastic responses can be found on a broad biogeographical scale across several latitudes (Osovitz and Hofmann, 2005) or on a small scale within one site or sites closely situated to each other (Helmuth and Hofmann, 2001). The aim of the current study was to investigate the physiological responses of different mussel species to environmental stressors at different scales.

M. galloprovincialis and *P. canaliculus* are major space occupiers on rocky shores around New Zealand (Menge *et al.*, 1999; Morton, 2004; Menge *et al.*, 2007). *P. canaliculus* dominates the low intertidal zone and *M. galloprovincialis* dominates the mid intertidal zone (Menge *et al.*, 1999; Morton, 2004; Menge *et al.*, 2007). It was anticipated that the two species would have different tolerances to stress due to the different localisations of the mussel species in the intertidal zone. Petes *et al.* (2007) showed that *P. canaliculus* is more susceptible to extreme thermal events than is *M. galloprovincialis* in the mid-intertidal zone. *P. canaliculus* is also competitively dominant to *M. galloprovincialis* in the low-intertidal zone (Paine, 1971; Menge *et al.*, 1999; Morton, 2004; Menge *et al.*, 2007). Despite these findings, the zonation between these two species of mussel in New Zealand is indistinct (Menge *et al.*, 1999; Menge *et al.*, 2007). The two species co-occur in a mixed band, illustrating that factors besides competition affect zonation, as suggested by Connell (1961). It is likely that there are differences in physiological tolerances between the two species that enable them to coexist in particular areas of the intertidal zone. Menge *et al*. (2007) investigated this zonation pattern with limited success. *P. canaliculus* was predicted to be the best (higher survival, growth and RNA:DNA ratio) at more exposed locations in the low shore while *M. galloprovincialis* was predicted to be the more competitive in protected habitats higher on the shore (Menge *et al.*, 2007). The predictions were supported by the experimental findings in some cases (e.g., *M. galloprovincialis* grew better at the

sheltered site), but not in others (e.g., *P. canaliculus* growth rate was highest at the protected site and there was no difference in mortality between zones at the sheltered site for *P. canaliculus*). Between-zone translocations of the two mussel species in the experiments for this chapter allowed for additional investigation into how physiological stress responses to varying habitats might differ between the two species and affect their zonational patterns.

Stress is frequently measured by the expression and regulation of heat shock proteins (HSPs), which are required to mitigate the effects of harmful conditions (Feder and Hofmann, 1999). HSP expression is by thermal stress and potentially other stressors In the absence of stress, HSPs can be present at low levels to chaperone the folding of nascent polypeptides (Hofmann and Todgham, 2010). HSPs refold damaged proteins and decrease further degradation during exposure to stressful conditions (Hofmann and Todgham, 2010). The magnitude of expression is a direct measure of the extent to which an organism is experiencing stress (Feder and Hofmann, 1999). There are several classes of HSPs (Lindquist, 1986; Gething, 1997; Feder and Hofmann, 1999); *hsp70*, *hsp90* and small *hsps* (*hsp24*) are studied here due to previously published reports of their expression in mussels responding to environmental stress (Place *et al.*, 2008; Lockwood *et al.*, 2010).

In addition to HSPs, there are other stress-induced genes that function to prevent or decrease damage to the cell. *E74-like factor 2* (*elf2*) and *butyrate response factor 2* (*tis11d*) are two such examples. Elf2 is upregulated in response to a variety of stressors, including hypoxia and cytokine stress (Sharrocks, 2001; Christensen *et al.*, 2002; Zhang *et al.*, 2007). *Tis11d* is involved in transcriptional regulation during stress (Hudson *et al.*, 2004; Murata *et al.*, 2005; Baou *et al.*, 2009). Both of these genes were upregulated in the mussel *M. californianus* at stressful sites in the Pacific North West (Place *et al.*, 2008).

The physiological tolerances of *M. galloprovincialis* and *P. canaliculus* were investigated here by using RT-qPCR to assess expression of stress response genes (*hsp24, hsp70, hsp90, elf2, tis11d*). Growth and survival were also measured and linked to temperature and emersion times (time in air). The molecular analyses used in this chapter were described in detail in Chapter Two.

These experiments were done at several sites around the South Island of New Zealand to enable comparisons among different coastal conditions as well as

for site replication (Chapman, 1986). Differences in water quality and food availability between the east and west coasts are likely to affect intertidal animals (Menge *et al.*, 1999; Menge *et al.*, 2002; Menge *et al.*, 2003). A description of oceanographic conditions around the South Island is available in **Section 1.4**.

Studies that test stress responses of filter-feeders are generally done on adult mussels where some selection has already occurred on the population. Large adults have already survived through smaller juvenile stages to reach adulthood. During low tide, mussels retain water inside their shells, helping to keep body temperature and desiccation to a minimum. Thus, animals of different sizes may be affected in different ways over their lifetimes by the same stressors. The differences in response between small and large mussels were also tested. It was recognised that their surface-to-volume ratios are different, which may affect internal heat stress.

Hypotheses tested in Chapter Three:

- Mussels translocated from their native intertidal zone into a different intertidal zone will show evidence of differential stress over the course of summer, particularly in the mid-tidal zone;
- *Mytilus galloprovincialis* (a true intertidal-zone species) will have a lower intensity of stress response than *Perna canaliculus* (a low intertidal-subtidal-zone species);
- Small mussels will have an enhanced stress response relative to large mussels;
- There will be a higher stress response on the east coast of New Zealand, where water nutrients are generally lower, than on the west coast.

3.2 Methods

3.2.1 Study sites

The experiment was set up in the summer of 2008/2009 to have replicate sites on the east and west coast of the South Island (**Figure 3.1**) to allow regional comparisons of mussel responses. The east and west coasts differ in their oceanographic regimes (Schiel, 2004), nutrients, mussels recruitment and growth (Menge *et al.*, 2003; Bracken *et al.*, 2012). The two west coast sites were

Woodpecker Bay (WB) and Nine Mile Beach (NMB; **Figure 1.3** and **1.4**). The east coast sites were Box Thumb (BT) and Timaru (TIM; **Figure 1.7** and **1.8**).

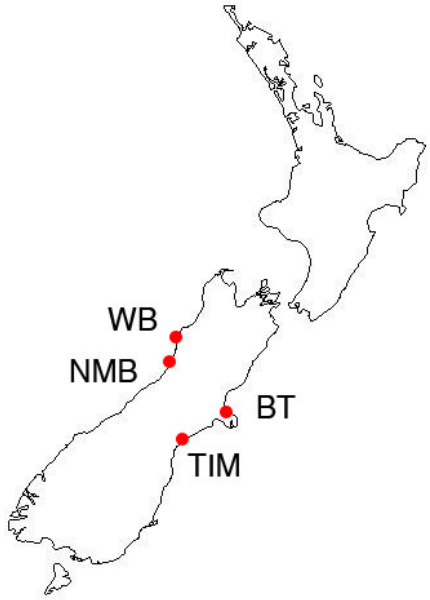


Figure 3.1 Map of field site locations for the translocation experiment. WB, Woodpecker Bay; NMB, Nine Mile Beach; BT, Box Thumb; TIM, Timaru.

3.2.2 Temperature recording

At each site, two Tidbit™ temperature loggers (Onset Computer Corp., Pocahassett, MA, USA) were deployed in the intertidal zone: one in the low-zone within a bed of *P. canaliculus* and the other in the mid-zone within a bed of *M. galloprovincialis* (Helmuth and Hofmann, 2001; Menge *et al.*, 2003; Petes *et al.*, 2008). The mid and low intertidal zones were defined by the species distribution; the mid-zone is dominated by *M. galloprovincialis* while the low-zone is dominated by *P. canaliculus*. Surface temperature was recorded every 10 minutes for the duration of the experiment. Tidbit™ temperature loggers are capable of measuring a range of temperatures from −4˚C to 38˚C. Temperature data were coupled with tide tables to separate air and water temperatures. The daily emersion time at each site was calculated using the decoupled air and water temperature readings. Unfortunately, the low-zone data logger at NMB failed.

A temperature stress index (TSI) was created to allow for examination of the effects of air temperature and emersion time together. Daily minimum, maximum and average temperatures are typically used when studying intertidal organisms.

The temperatures commonly reported do not differentiate between air and water temperatures (Braby and Somero, 2006; Henkel *et al.*, 2009; Kuo and Sanford, 2009). However, the air temperature the mussels experience during exposure at low tide is the most stressful time (Connell, 1961; Connell and Orias, 1964).

The TSI is the sum of temperatures during air exposure. A higher TSI would result from either longer air exposure (more temperature readings to sum) or hotter temperatures during air exposure (greater temperature values to sum).

3.2.3 Translocation experiment

The experimental design of this study consisted of between-zone translocation of mussels (mid and low-intertidal zone; **Figure 3.2**). Two size classes (large and small) were used for each of two mussel species (*M. galloprovincialis* and *P. canaliculus*). Each zone had translocated clumps of mussels of each species and each size (n = 3 clumps for each treatment). Across all sites, the mid-zone and low-zones differed by 0.50 ± 0.15 m in tidal elevation (**Table 3.1**). Control translocations were done such that mussels collected from one zone were translocated back into that same zone. This study was replicated between two regions (east and west coast) with two sites in each region (**Figure 3.1**).

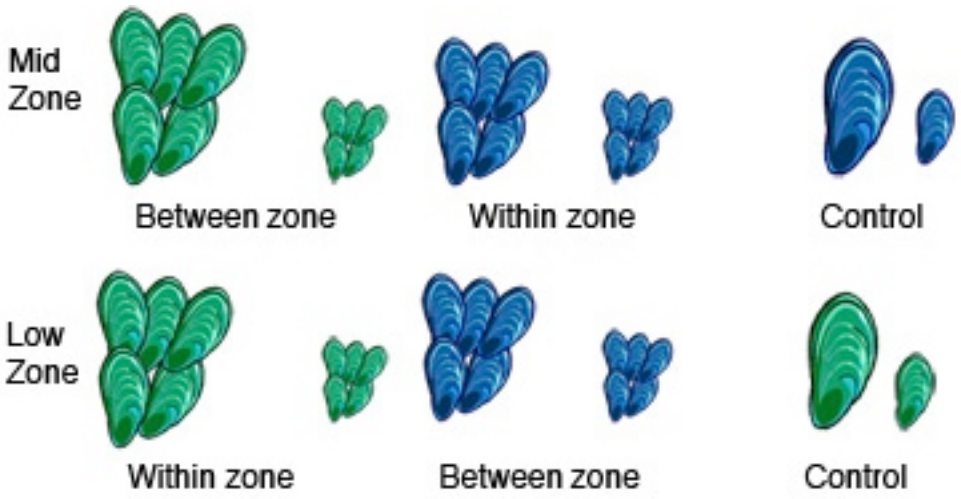


Figure 3.2 Diagram of experimental design. *M. galloprovincialis* is in blue and *P. canaliculus* is in green. Large and small *M. galloprovincialis* were translocated from mid-zone to mid-zone (control translocation) and from mid-zone to low-zone. Large and small *P. canaliculus* were translocated from low-zone to low-zone (control translocation) and from low-zone to mid-zone. Control untransplanted mussels were collected from undisturbed regions of the mussel bed.

Table 3.1 Tidal elevation between experimental plots (metres (m) above low tidal datum, mean ± SD).

	WB	NMB	BT	TIM
Mid-zone	2.58 ± 0.20	1.20 ± 0.05	2.19 ± 0.07	2.50 ± 0.17
Low-zone	1.94 ± 0.13	0.82 ± 0.03	1.69 ± 0.12	2.04 ± 0.08
Difference	0.64	0.38	0.50	0.47

This study was done in the austral summer, from November 2008 to April 2009. The experiments at NMB and WB were installed on 11 November 2008 and 13 November 2008 respectively, while the experiments at BT and TIM were installed on 18 November 2008 and 16 December 2008.

Mussels were translocated within and between intertidal zones following previously reported methods (Chapman, 1986; Menge *et al.*, 1994; Honkoop *et al.*, 2003; Blanchette *et al.*, 2007; Menge *et al.*, 2007; Menge *et al.*, 2008). *M. galloprovincialis* and *P. canaliculus* were collected haphazardly from their respective intertidal zone by gently cutting the byssal threads that attached them to the substratum. *M. galloprovincialis* and *P. canaliculus* occupy the mid and low-intertidal zones respectively. To test for variation in stress responses due to mussel size, two size classes were collected, 'small' (20-40 mm) and 'large' (40-70 mm) for each species from their normal intertidal zone. The lower limit on size class was set to prevent subtidal predation by crabs and fish. It has been shown that mussels larger than 15 mm were not prey items for common subtidal predators (Rilov and Schiel, 2006b; a). Mussels can grow larger than 70 mm but the upper limit was set to allow for mussels to continue growing during the experiment since growth was one of the measured factors. Each mussel was notched with a file in the posterior tip of the shell. Care was taken that the notch was shallow to avoid damaging the mantle. A scar formed in notches, allowing measurement of subsequent growth. Small and large *M. galloprovincialis* and *P. canaliculus* were then sorted into groups of 25 and placed ventral side down in the middle of the mid or low-zone mussel bed onto bare rock that had been cleared of biota. Clumps of small and large transplanted mussels of each species were placed in both the low and mid-intertidal zone. The mussels were then covered with Vexar plastic mesh that was held to the rock using plastic washers and lag screws placed into pre-drilled holes (**Figure 3.3**). After allowing one month for mussels to attach to the rock, the mesh

was loosened, enabling the animals to orient themselves while still protecting them from being dislodged from wave forces or other disturbances. After a second month, the mesh was removed after ensuring all mussels were attached.

Figure 3.3 Translocated mussels re-attaching to the rock under Vexar mesh.

For molecular analyses, gill tissue samples were collected from each experimental plot at three time points: November 2008 (T_0), March 2009 ($T_{0.5}$) and April 2009 (T_f) (n = 3 for each treatment where possible). Mussels were opened by severing the adductor muscle and the gill was removed and placed into a 1.5 ml tube. The tubes were dropped into liquid nitrogen to flash-freeze the tissue, then brought back to the laboratory and stored at −80˚C until processed. Control tissue samples were collected from wild-type animals that were living unperturbed in a natural mussel bed.

The number of surviving mussels remaining in each plot was surveyed at monthly intervals. Missing mussels or gaping shells that remained in the plots were considered dead. At all sites, the growth of all surviving translocated and notched mussels was measured with Vernier calipers at the end of the experiment.

3.2.4 Molecular methods

Mussel tissue was prepared for RT-qPCR by RNA extraction and cDNA synthesis through various methods described in Chapter Two. Tissue from 1-3 animals was pooled for RNA extraction, depending on the number of samples that

could be collected, which was sometimes influenced by mortality in the field. RT-qPCR reactions were run in triplicate. Five target genes in *M. galloprovincialis* (*hsp24, hsp70, hsp90, elf2* and *tis11d*), and one target gene in *P. canaliculus* (*hsp70*) were assessed for relative expression levels between the experimental treatments. *P. canaliculus* was limited to only one target gene due to limited sequence availability in public databases. The RT-qPCR reactions for *elf2* and *hsp90* failed for samples from WB and due to time limitations could not be repeated. As a result, regional comparisons in gene expression responses for these genes (*hsp90*, *elf2*) were not possible.

3.2.5 Data analyses

All data analyses were done in R statistical software (Version 2.14.1; R Development Core Team, 2011). Differences in the proportion of mussel survival between the different factors were tested with a Generalised Linear Model (GLM). The GLM used a logit link function and quasibinomial distribution to account for overdispersion. The test factors were mussel species (*M. galloprovincialis* vs. *P. canaliculus*), size (large vs. small), tidal zone (mid vs. low) and region (east vs. west), which were analysed as fixed factors. Sites were analysed as a random factor nested within region. A likelihood ratio test with a Chi-Squared distribution was run on the GLM results. *Post hoc* multiple comparisons were performed on the significant GLM results with probabilities adjusted using Tukey HSD to control for multiple tests.

To establish if temperature was a significant factor in survival, a second GLM was run biasing it to assume that temperature could account for all the variation in survival. Temperature was a continuous variable, measured with one data logger in each zone at each site. As a result, temperature and region/site and zone co-vary. A linear regression with the residuals of both models was done to see if average daily maximum temperature could explain significant variation in the original model. Using this two-step approach, I was able to assess the effect of temperature on mussel survival in a robust manner.

Growth analyses were done using a Generalised Linear Model with a Tweedie distribution with $p = 1.2$ and a log-link function to account for overdispersion and zeros in the dataset (Shono, 2008). Multiple *post hoc*

comparisons were performed on significant GLM interactions using Tukey HSD to control for multiple tests.

To determine if temperature was a significant predictor for mussel growth, a second GLM was run with temperature as the only factor. To compare the fit of the two models, an AIC was done and the delta AIC was calculated (Burnham and Anderson, 2002). The *Akaike* weight was used to show how well the two models (temperature only, and everything but temperature) fit the data set. This process was also used to test for a role of emersion time and TSI on mussel growth.

For the RT-qPCR data, a modified Pfaffl equation was used to determine fold change values (**Equation 2.2**) (Pfaffl, 2001; Hellemans *et al.*, 2007). The equation was modified to accommodate three reference genes. For each gene, log-transformed fold change relative gene expression data was analysed with a three-way nested ANOVA testing for differences between site nested within region, zone, and size.

To test how the variability of survival and growth was accounted for by environmental factors, a non-parametric multivariate regression analysis was done using the Vegan package in R (Oksanen *et al.*, 2012). For the analyses with *M. galloprovincialis*, due to some failed RT-qPCR reactions resulting in an unbalanced dataset, only three genes (*hsp24, hsp70* and *tis11d*) could be included in the multivariate analyses. Initially, the significance of each explanatory variable (region, site, zone, size, temperature, emersion time and TSI) was determined. The response variables (survival, growth and gene expression) and explanatory variables were then fit to the ordination through 9999 permutations of the normalised predictor data, while conditional tests were done using 9999 permutations of residuals under the reduced model. All tests were based on Euclidian dissimilarities calculated among log transformed gene expression data. Principle coordinate analysis (PCoA) was used to visualise the relationships between global gene expression and temperature. The same analyses were used for *P. canaliculus* with growth, survival and *hsp70* expression as response variables.

3.3 Results

3.3.1 Size class

The mean mussel length for each size class and species at each site is reported in **Table 3.2**. There were no significant differences between sites for the size classes of either species (one-way ANOVA, $p < 0.05$; **Table 3.3**, **Table 3.4**, **Table 3.5**, **Table 3.6**).

Table 3.2 Size of mussels (length) used in experiments at the four different sites (mm, mean ± SD, n=25)

	BT	**TIM**	**WB**	**NMB**
Large *M. galloprovincialis*	53.71 ± 2.99	53.22 ± 3.04	53.86 ± 2.60	55.32 ± 3.09
Small *M. galloprovincialis*	32.13 ± 3.31	33.28 ± 3.14	31.81 ± 2.66	33.28 ± 2.95
Large *P. canaliculus*	55.13 ± 2.90	55.87 ± 3.43	57.02 ± 2.18	56.25 ± 3.10
Small *P. canaliculus*	28.67 ± 2.73	29.77 ± 2.87	28.26 ± 2.82	27.55 ± 3.93

Table 3.3 Small *M. galloprovincialis*, one-way ANOVA, mussel length at each study site ($p < 0.05$).

	SS	**df**	**MS**	**F**	***p*-value**
Site	97.7	3	32.56	1.781	0.156
Residuals	1755.4	96	18.29		

Table 3.4 Large *M. galloprovincialis*, one-way ANOVA, mussel length at each study site ($p < 0.05$).

	SS	**df**	**MS**	**F**	***p*-value**
Site	151.4	3	50.46	2.694	0.0503
Residuals	1798	96	18.73		

Table 3.5 Small *P. canaliculus*, one-way ANOVA, mussel length at each study site ($p < 0.05$).

	SS	**df**	**MS**	**F**	***p*-value**
Site	41.5	3	13.82	0.61	0.61
Residuals	2173.9	96	22.64		

Table 3.6 Large *P. canaliculus,* one-way ANOVA, mussel length at each study site ($p < 0.05$).

	SS	df	MS	F	*p*-value
Site	47.1	3	15.7	1.356	0.261
Residuals	1111.9	96	11.58		

3.3.2 Temperature

Daily maximum air temperatures were consistently higher at TIM than at the other three sites, with several temperature spikes in the mid-zone reaching the maximum measurable temperature of the Tidbit™ data logger at 38˚C throughout the summer (**Figure 3.4D; Figure 3.5**). There was considerable daily variation in the maximum temperatures at all sites although BT was the least variable (**Figure 3.4C**). The TIM site was the most variable and the hottest, BT was the coolest site with the lowest variation. Thermal environments at NMB and WB were similar to each other in terms of variation and maximum temperatures, although WB was slightly warmer. The low-zone temperature surpassed 30˚C on three occasions at TIM, but not at all at any other site (**Figure 3.4**). The average afternoon temperatures, the time of day with the most potential for thermal stress, between zones at BT barely differed (mean [±SE] difference, 0.07 ± 0.39˚C; **Figure 3.6**). The minimal differences in temperature between zones at BT could result from the smaller size of the rocky bench that may allow for typical wave action to splash up into the mid-intertidal zone, maintaining cooler temperatures for organisms (and that data logger). Other sites had larger between-zone temperature differences possibly resulting from larger distances between the translocated plots compared to BT.

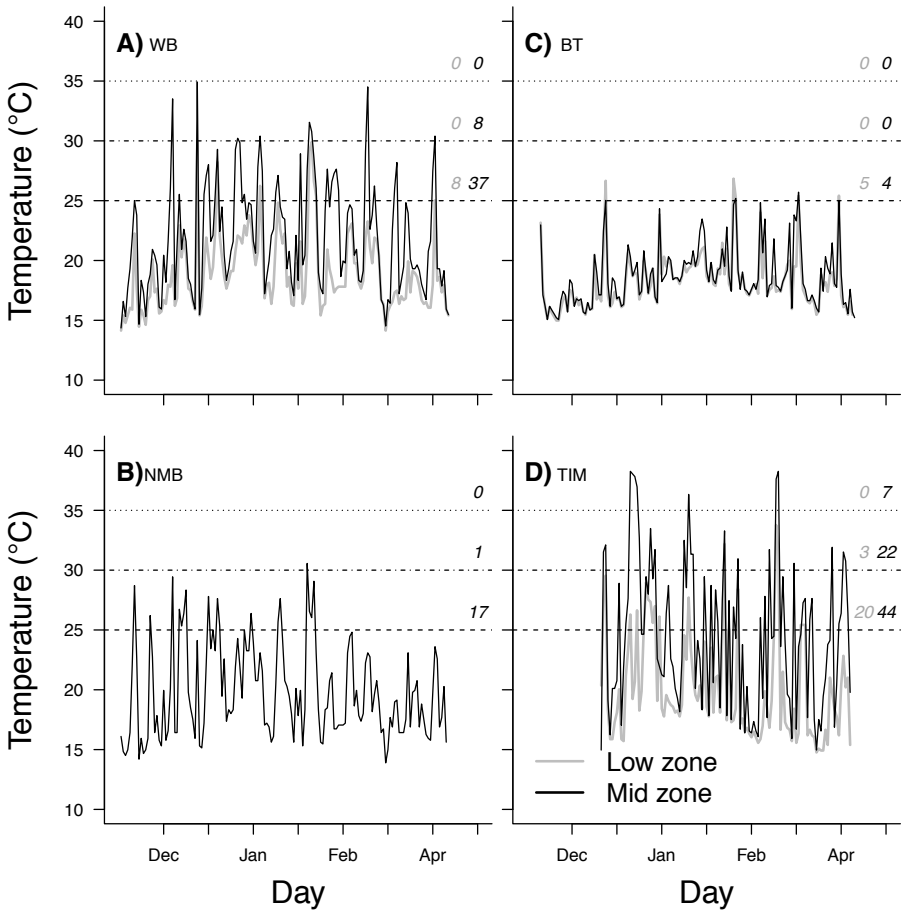


Figure 3.4 Daily maximum air temperature. Mid-zone (black lines) and low-zone (grey lines) at the four sites, A) Woodpecker Bay (WB); B) Nine Mile Beach (NMB); C) Box Thumb (BT); D) Timaru (TIM). Horizontal dashed lines and italicised numbers highlight the number of days that the temperature was above 25˚C, 30˚C or 35˚C at each site; black and grey text represent mid and low-zone respectively. The experimental duration at each site ranged from 112-147 days from December 2008 –April 2009.

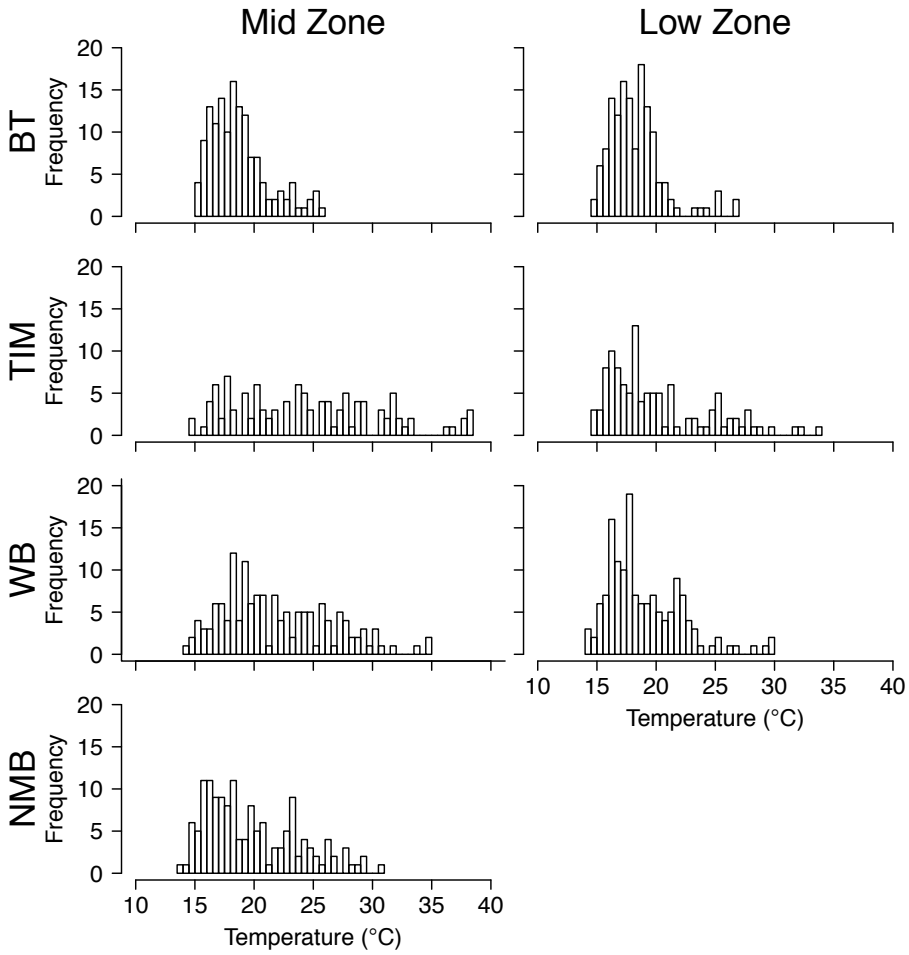


Figure 3.5 Frequency histogram of daily maximum air temperature for each site and intertidal zone for the duration of the experiment from December 2008-April 2009. BT: Box Thumb, TIM: Timaru, WB: Woodpecker Bay, NMB: Nine Mile Beach.

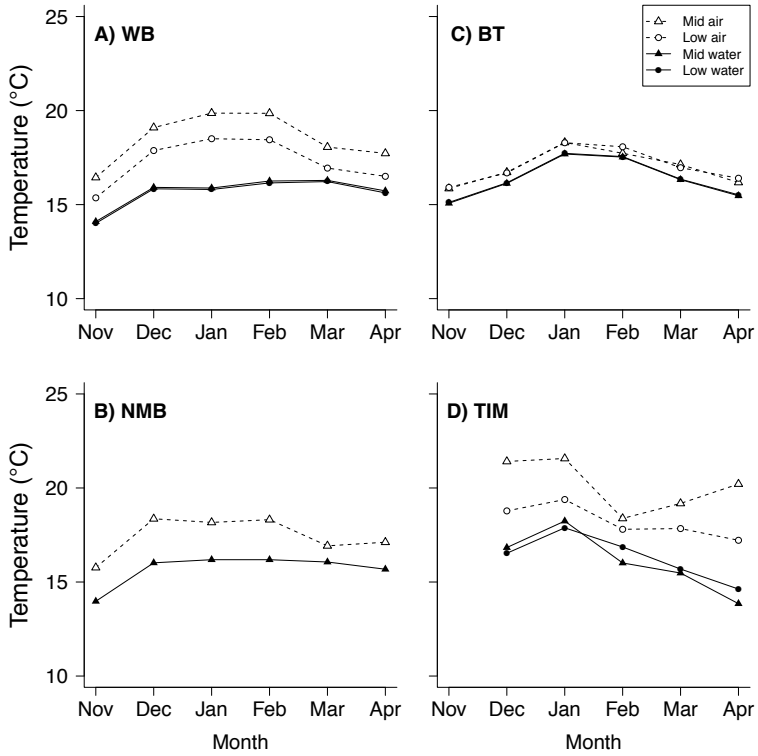


Figure 3.6 November-April, 2008-2009, monthly mean high temperatures from the mid-zone logger (triangles) and low-zone logger (circles) at each of the four sites. The mean monthly water temperature in each zone is also plotted. A) Woodpecker Bay (WB); B) Nine Mile Beach (NMB); C) Box Thumb (BT); D) Timaru (TIM). The low-zone temperature logger failed at NMB.

3.3.3 Emersion

The mean (± 1 SD) hours per day that mussels were exposed to air varied by several hours among sites (**Table 3.7**). TIM had the longest emersion time in the mid-zone and the greatest difference between tidal zones (**Figure 3.7**). BT and WB had similar time differences between the low and mid-zone emersion.

Table 3.7 Daily emersion time in hours for mussels in the mid and low intertidal zones at each site (means ± SD), NA: not available.

	WB	**NMB**	**BT**	**TIM**
Mid-zone	12.53 ± 0.51	11.13 ± 0.82	11.15 ± 0.54	15.94 ± 0.72
Low-zone	9.60 ± 1.13	NA	8.21 ± 0.91	7.12 ± 1.12

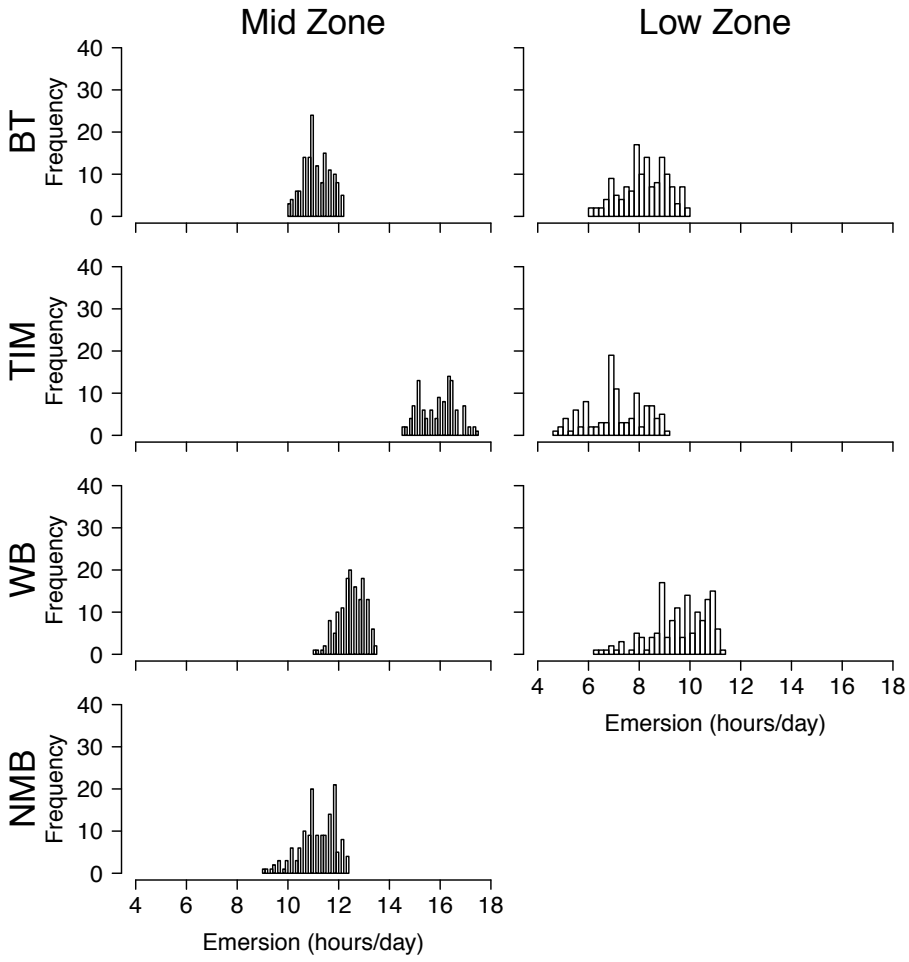


Figure 3.7 Frequency histogram of daily emersion time (h) for each site and intertidal zone for the duration of the experiment from December 2008-April 2009. Emersion is the hours per day of air exposure. BT: Box Thumb, TIM: Timaru, WB: Woodpecker Bay, NMB: Nine Mile Beach.

3.3.4 Temperature stress index (TSI)

The TIM site had the greatest difference in TSI between intertidal zones, while BT had the least (**Table 3.8**). The mid-zone TSI at WB was similar to TIM while the low-zone at WB was higher. Overall, the TSI for WB suggests it should be the most stressful site. However, the high temperature and longer emersion in the TIM mid-zone suggests it would have the greatest environmental stress, while low temperatures and emersion time at BT suggest it should be the least stressful.

Table 3.8 Temperature stress index (TSI) for mussels in the mid and low intertidal zones at each site, NA: not available. TSI is the sum of temperature readings during air exposure.

	WB	**NMB**	**BT**	**TIM**
Mid-zone	765	634	587	770
Low-zone	541	NA	433	284

3.3.5 Survival

Survival does not appear to have been affected in this experiment by abiotic factors such as temperature and emersion or the combination of the two (TSI). All three of those factors were non-significant for both species in all treatments (GLM, temperature: $p = 0.835$; emersion time: $p = 0.200$; TSI: $p = 0.071$).

3.3.5.1 *M. galloprovincialis* survival

At all sites, the survival of large *M. galloprovincialis* mussels in the mid-zone was better than in the low-zone (**Figure 3.8**). If survival was influenced primarily by temperature, better survival would be expected in the low-zone. It is likely that a factor other than temperature or emersion is driving survival of large *M. galloprovincialis*. The east coast sites (BT and TIM) had higher mortality in both size classes of *M. galloprovincialis* than the west coast sites (WB and NMB; **Figure 3.8**). TIM was warmer than the other sites (**Figure 3.4**), it seems likely that temperature may have had an effect on survival at this specific site despite the fact that temperature does not explain survival at other sites and was not significant in the GLM. The GLM with all the factors (region, site, zone, size, species) showed several significant interactions among the different factors ($p < 0.05$, pseudo $R^2 = 0.52$; **Table 3.9**). Mortality of small *M. galloprovincialis* was higher in the low-zone than in the mid-zone at BT and NMB.

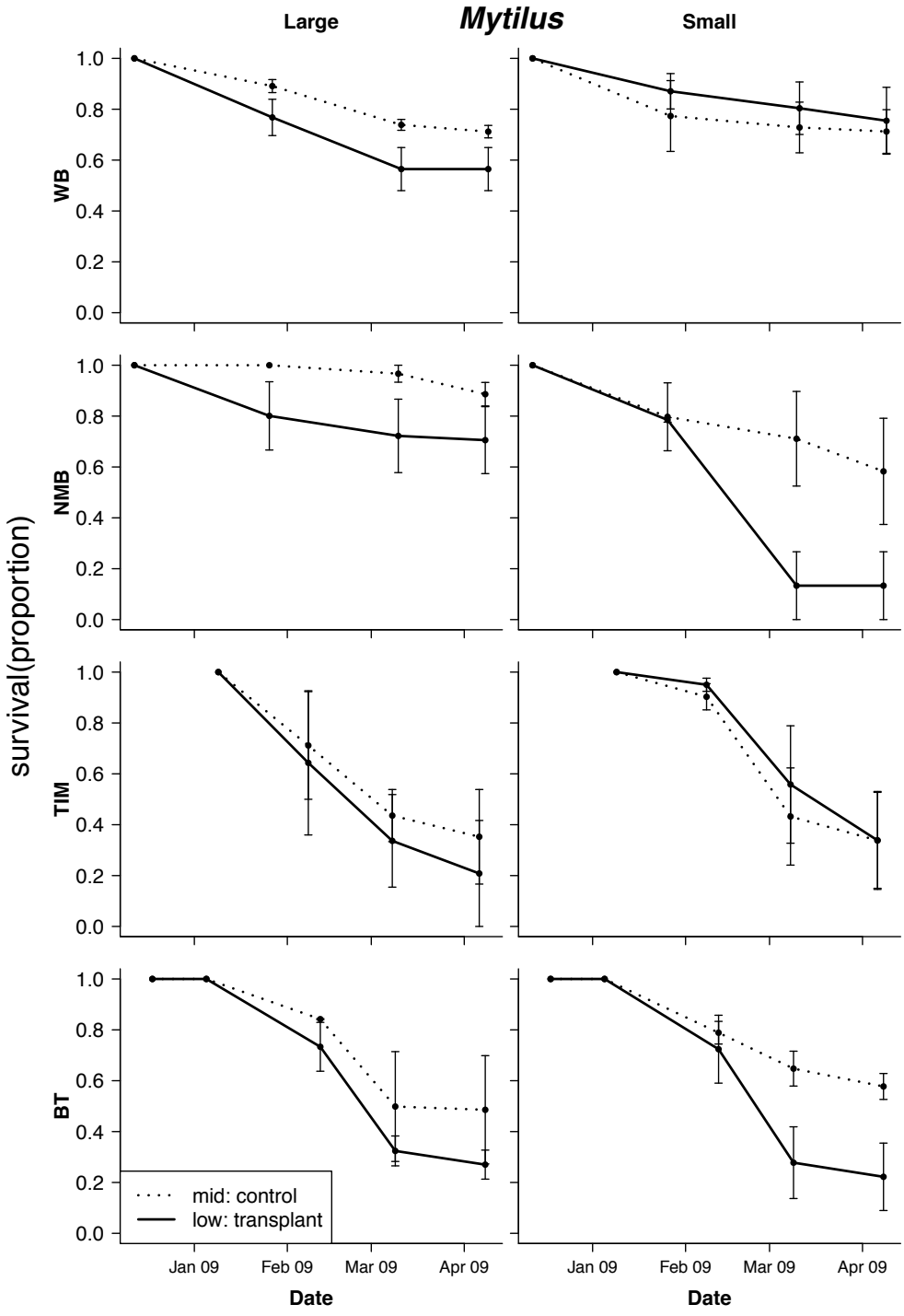


Figure 3.8 Proportion of survival in transplanted *M. galloprovincialis* throughout the course of the field experiment in summer 2008/2009. Large (left column) and small (right column) mussels at each site (WB, Woodpecker Bay, NMB, Nine Mile Beach; TIM, Timaru; BT, Box Thumb). Mean survival proportion (± SE) plotted for each zone (dotted line, mid-zone; solid line, low-zone) at approximately monthly intervals.

3.3.5.2 *P. canaliculus* survival

P. canaliculus survival trends were more varied than those of *M. galloprovincialis* (**Figure 3.9**). In only one of four sites for large mussels and two of four cases for small mussels was survival better in the low tidal zone. At WB, large and small mussels had similar survival (80.54 ± 6.94%, 81.40 ± 6.84% respectively) in the low-zone, while survival of large and small sizes in the mid-zone was only 41.08 ± 19.47%, and 34.11 ± 20.01% (**Figure 3.9**). At NMB, survival of large *P. canaliculus* differed little between zones (low: 47.92 ± 27.08%; mid: 42.50 ± 15.07%), but most small mussels in the low-zone died (98.61 ± 1.39% mortality; **Figure 3.9**). At TIM, mid-zone survival of large and small *P. canaliculus* was similar (43.75 ± 43.75% and 40.04 ± 21.86%). Size classes differed greatly in survival in the low-zone, however, with 91.11 ± 8.89% mortality of large mussels and 23.02 ± 7.57% mortality of small mussels (**Figure 3.9**). At BT, overall survival was high in both zones (**Figure 3.9**).

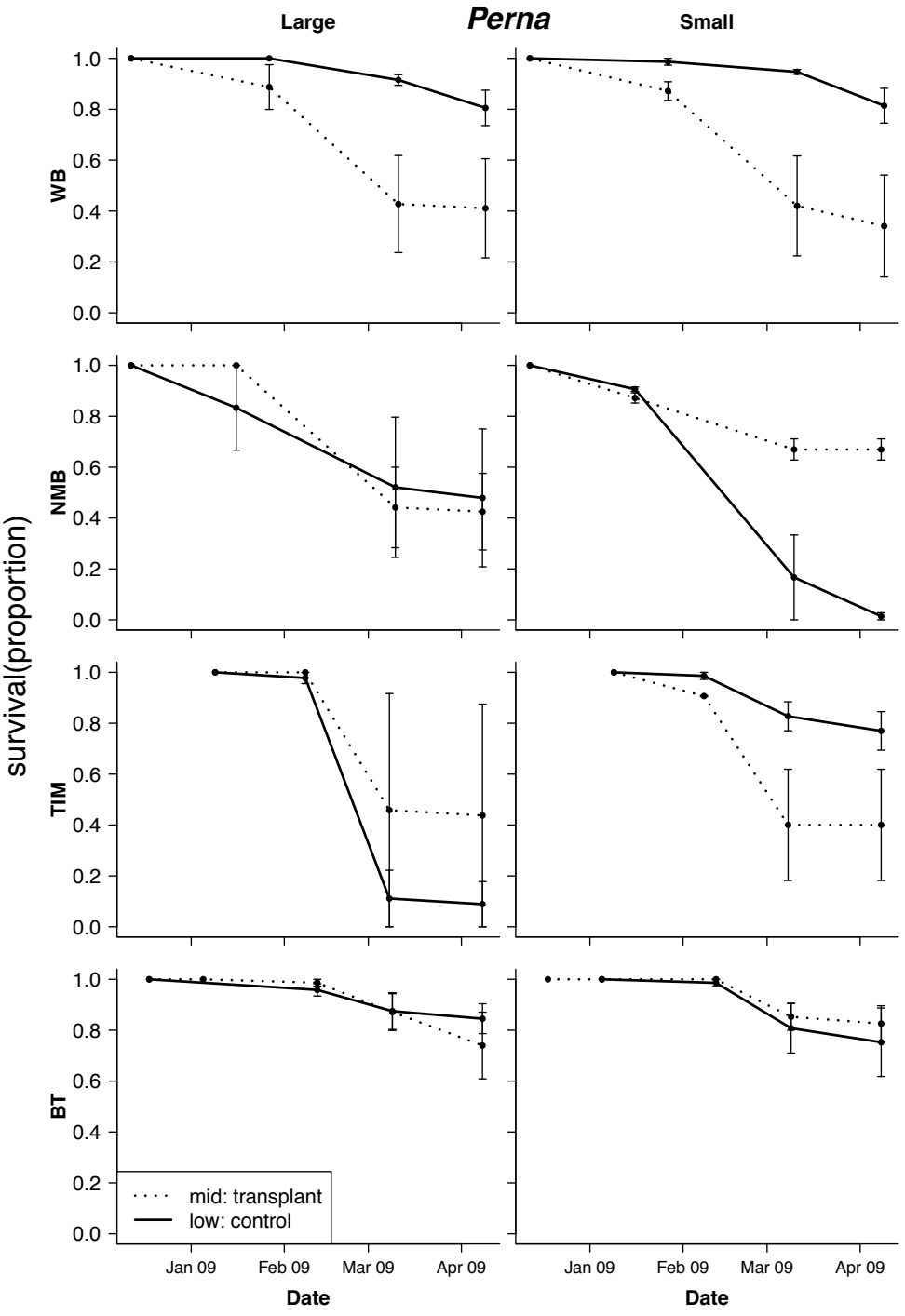


Figure 3.9 Proportion of survival in transplanted *P. canaliculus* throughout the course of the field experiment in summer 2008/2009. Large (left column) and small (right column) mussels at each site (WB, Woodpecker Bay, NMB, Nine Mile Beach; TIM, Timaru; BT, Box Thumb). Mean survival proportion (± SE) plotted for each zone (dotted line, mid-zone; solid line, low-zone) at approximately monthly intervals.1

There was a significant three-way interaction in survival, site(region) × zone × size (LRT $\chi^2_1 = 6.385$, $p = 0.0155$; **Table 3.9**; **Figure 3.10A**). In general, regardless

of species, small mussels in the low-zone at NMB survived poorly as did large mussels in the low-zone at TIM. At NMB, a large amount of sand moved into the intertidal region from offshore during the course of the experiment, smothering many of the plots in the low-zone (**Figure 3.11**). Greater mortality of small mussels at NMB suggests that they were more vulnerable to sand burial than the large mussels. The low-zone mussels of both sizes survived marginally better at WB, while the other sites did not have any notable patterns. Despite the predictions of a size advantage for large mussels, there was little evidence that size conferred an advantage to mussel survival.

Table 3.9 Quasibinomial GLM analysis of deviance table for mussel survival. Test factors in bold were significant ($p < 0.05$).

	LR Chisq	**df**	**Pr(>Chisq)**
Region (east vs. west coast)	**4.6526**	**1**	**0.0310**
Size (large vs. small)	0.4486	1	0.5030
Zone (mid vs. low)	0.0621	1	0.8032
Species (*Myt* vs. *Per*)	**8.886**	**1**	**0.0029**
Site(region)	3.9739	2	0.1371
Region × size	0.1343	1	0.7140
Region × zone	**6.0681**	**1**	**0.0138**
Size × zone	1.1545	1	0.2826
Region × species	**14.3288**	**1**	**0.0002**
Size × species	0.0004	1	0.9835
Zone × species	**7.3348**	**1**	**0.0068**
Site(region) × size	0.8347	2	0.6588
Site(region) × zone	0.1541	1	0.6946
Region × size × zone	0.0629	1	0.8020
Site(region) × species	1.3515	2	0.5088
Region × size × species	0.0153	1	0.9016
Region × zone × species	0.6405	1	0.4235
Size × zone × species	0.0721	1	0.7884
Site(region) × size × zone	**6.385**	**1**	**0.0115**
Site(region) × size × species	2.7068	2	0.2584
Site(region) × zone × species	0.3973	1	0.5285
Region × size × zone × species	0.322	1	0.5704
Site(region) × size × zone × species	1.6136	1	0.2040

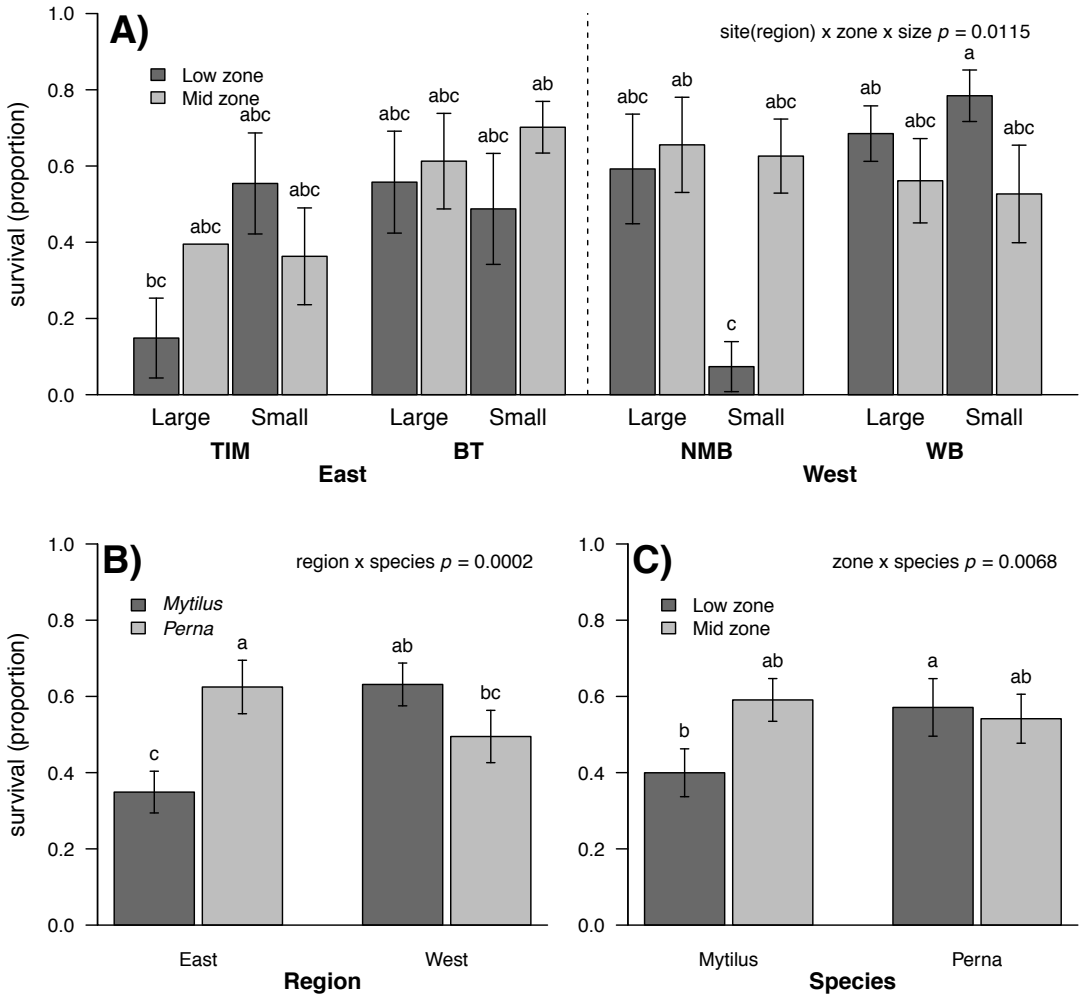


Figure 3.10 Proportion of survival (mean [± SE]) at the end of the translocation experiment. A) Survival of large and small mussels of both species combined (*M. galloprovincialis* and *P. canaliculus*) in different intertidal zones at the four sites (WB, Woodpecker Bay, NMB, Nine Mile Beach; TIM, Timaru; BT, Box Thumb; Tukey HSD, $p < 0.05$). B) Survival of both species on the east and west coasts (Tukey HSD, $p < 0.05$). C) Between intertidal zone survival of *M. galloprovincialis* and *P. canaliculus* (Tukey HSD, $p < 0.05$). Matching letters denote non-significant differences.

Figure 3.11 Mussels smothered by sand in the low-zone at NMB.

There was also a significant region × species interaction in survival (LRT χ^2_1 = 14.3288, $p < 0.0002$; **Figure 3.10B**). *M. galloprovincialis* survival was better on the west coast than on the east coast (Tukey HSD $p = 0.0025$). *P. canaliculus* however, survived significantly better on the east coast (Tukey HSD $p = 0.0485$). On the east coast, *P. canaliculus* survival was significantly better than *M. galloprovincialis* (Tukey HSD $p = 0.0001$). On the west coast there were no significant differences in survival between species (Tukey HSD $p = 0.30$), although *M. galloprovincialis* fared better than *P. canaliculus*.

In a different two-way interaction, there were differences in survival between species in different zones (LRT $\chi^2_1 = 7.3348$, $p = 0.0068$). *P. canaliculus* in the low-zone survived better than *M. galloprovincialis* (Tukey HSD $p = 0.052$; **Figure 3.10C**). *P. canaliculus* survived equally well in both zones (Tukey HSD $p = 0.98$). *M. galloprovincialis* survival between intertidal zones was not significantly different (Tukey HSD $p = 0.13$). In all cases, there was a strong effect of site within region for survival.

3.3.6 Growth

As anticipated, large mussels grew very little over the course of the experiment compared to small mussels (**Figure 3.12**). Mussels of both species grew more in the low-zone, which is most prominent in *P. canaliculus* (**Figure 3.12**). Three different three-way interactions in a GLM were significant (**Table 3.10**). The

average daily maximum temperature GLM was statistically significant ($p < 0.05$, pseudo $R^2 = 0.27$, AIC = 1863.834, *Akaike* weight ~ 0) but had very little support in the data compared to the full model without temperature ($p < 0.05$, pseudo $R^2 = 0.66$, AIC = 1869.706, *Akaike* weight ~ 1). Similarly, neither emersion time ($p < 0.05$, pseudo $R^2 = 0.19$, AIC = 1908.908, *Akaike* weight ~ 0) or TSI ($p < 0.05$, pseudo $R^2 = 0.15$, AIC = 2229.662, *Akaike* weight ~ 0) significantly supported the variability in growth.

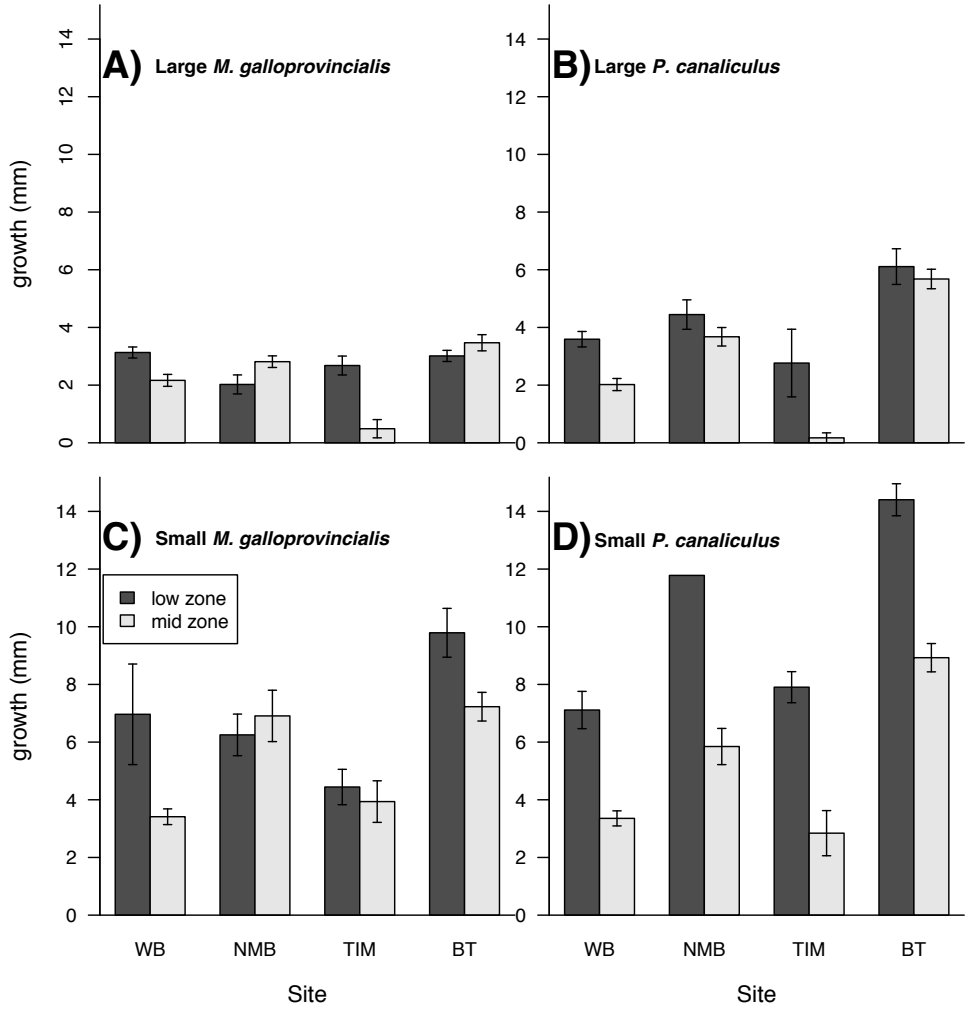


Figure 3.12 Total linear growth of transplanted mussels at the end of the translocation experiment, which lasted from Nov 2008-Apr 2009. Dark and light bars represent the low and mid intertidal zones respectively. A) Large *M. galloprovincialis,* B) Large *P. canaliculus,* C) Small *M. galloprovincialis,* D) Small *P. canaliculus.*

Table 3.10 Quasibinomial GLM analysis of deviance table for mussel growth. Test factors in bold were significant ($p < 0.05$)

	LR Chisq	df	Pr(>Chisq)
Region (east vs. west coast)	**58.11**	**1**	**<0.0001**
Zone (mid vs. low)	**81.48**	**1**	**<0.0001**
Size (large vs. small)	**344.69**	**1**	**<0.0001**
Species (*Myt* vs. *Per*)	**51.18**	**1**	**<0.0001**
Site(region)	**228.71**	**2**	**<0.0001**
Region × zone	0.83	1	0.3623
Region × size	**6.29**	**1**	**0.0122**
Zone × size	**4.91**	**1**	**0.0266**
Region × species	**6.49**	**1**	**0.0108**
Zone × species	**19.68**	**1**	**<0.0001**
Size × species	1.94	1	0.1634
Site(region) × zone	**25.98**	**2**	**<0.0001**
Site(region) × size	**13.93**	**2**	**<0.0001**
Region × zone × size	0.03	1	0.8681
Site(region) × species	**6.5**	**2**	**0.0388**
Region × zone × species	0.17	1	0.6819
Region × size × species	0.6	1	0.4404
Zone × size × species	0.09	1	0.7654
Site(region) × zone × size	**29.84**	**2**	**<0.0001**
Site(region) × zone × species	**9.38**	**2**	**0.0092**
Site(region) × size × species	**8.65**	**2**	**0.0132**
Region × zone × size × species	0.4	1	0.5279
Site(region) × zone × size × species	0.11	2	0.9481

In the site(region) × zone × size ($\chi^2_2 = 29.84$, $p < 0.0001$) interaction, large mussels at TIM grew the least and at BT small mussels in the low-zone grew the most of the entire experiment (**Figure 3.13**). Small mussels grew best in the low-zone; significantly so at three of the four sites (TIM, BT and WB; **Figure 3.13A**).

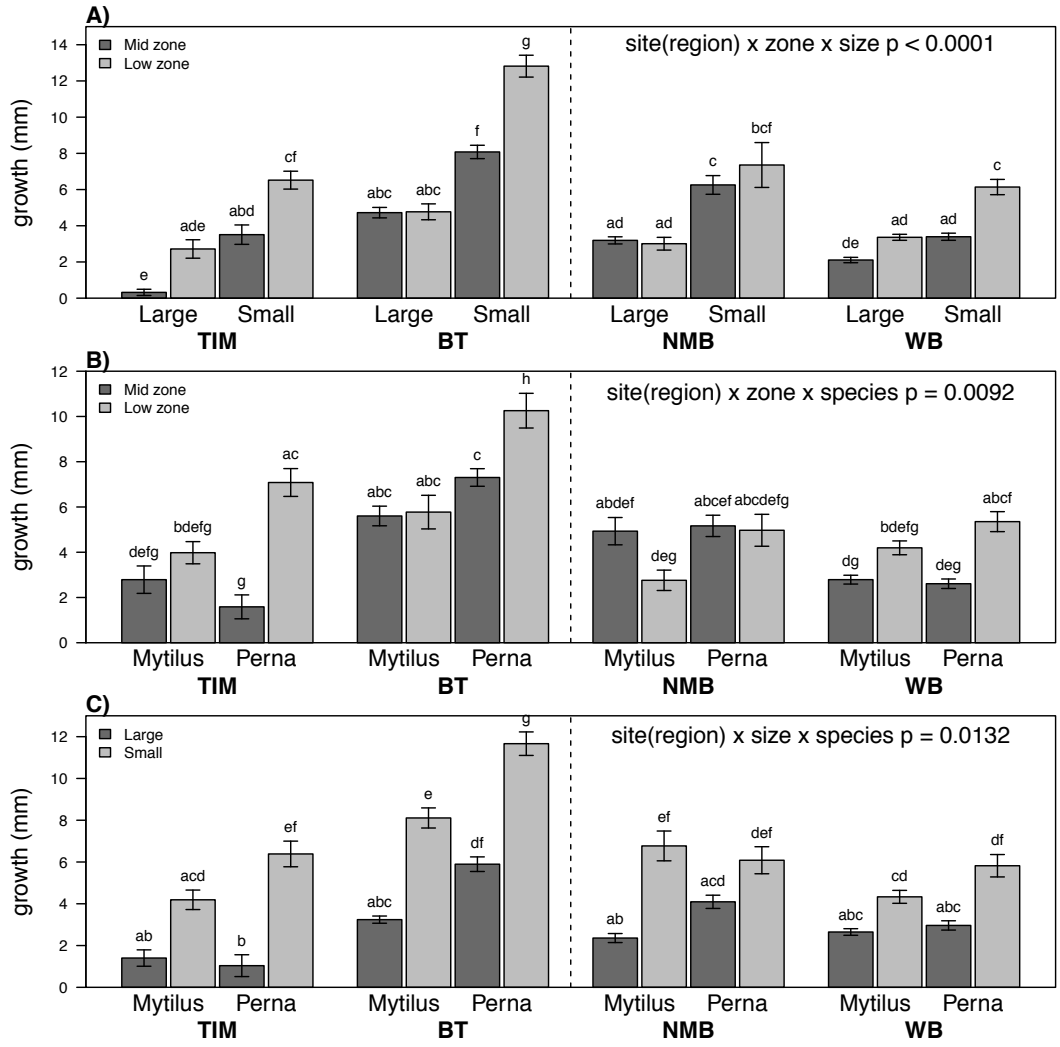


Figure 3.13 Mussel growth (mm; mean ± SE) at the end of summer of 2008/2009 after five months of translocation. A) Growth at each field site, for each species (*M. galloprovincialis* and *P. canaliculus*) in the low and mid intertidal zones. B) Growth at each field site for large and small mussels in the low and mid-zone. C) Growth at each field site for large and small mussels of each species (*M. galloprovincialis* and *P. canaliculus*). Matching letters indicate non-significant differences (Tukey HSD, $p < 0.05$).

In the site(region) × zone × species interaction ($\chi^2_2 = 9.38$, $p = 0.0092$), the multiple comparison significance was complex (**Figure 3.13B**). At the two east coast sites (BT, TIM), low-zone *P. canaliculus* grew the most, then *P. canaliculus* in the mid-zone and *M. galloprovincialis* in both zones. There tended to be more growth at BT than the other sites, but this was not necessarily significant.

In the site(region) × size × species interaction ($\chi^2_2 = 8.65$, $p = 0.0132$), the small mussels grew more than the large mussels for both species at all sites and this was significant in all cases except growth of *P. canaliculus* at NMB (**Figure**

3.13C). At three of four sites, small *P. canaliculus* grew significantly more than small *M. galloprovincialis*. NMB, the only site where this is not the case, had slightly more growth in *M. galloprovincialis* but not significantly so.

3.3.7 Gene expression

The physiological effect of between-zone translocations on gene expression of stress response proteins was assessed using RT-qPCR, with five genes as candidate biomarkers. Expression data were normalised with three different reference genes (*18S, 28S* and *actin),* and compared to calibrator and control samples for each time point. Gene expression from mid-summer and the end of summer collections did not differ for any of the six target genes (one-way ANOVAs, $p < 0.05$) and were therefore pooled for the analyses. In the few cases where error bars are missing on bar plots, this was due to mortality in the field limiting the number of individuals that could be collected for gene expression analyses.

hsp24

Gene expression of *hsp24* in *M. galloprovincialis* was significantly affected by region, site, zone and size ($F_{2, 12} = 6.45$, $p = 0.0125$, Tukey HSD, $p < 0.05$; **Table 3.11**, **Figure 3.14**). In general, gene expression of *hsp24* was downregulated at three of the four sites. Fold change tended to be greater in small than in large mussels. Fold change was enhanced through either up or downregulation in *M. galloprovincialis* that were transplanted from their native mid-zone to the low-zone, although not significantly so. Of particular interest was the TIM site, which had by far the highest temperatures and longest emersion times. At this site, small mussels strongly upregulated *hsp24* in both the mid and low-zone (mean ± SE, mid: 246 ± 158.15-fold, low: 414.45 ± 171.70-fold;), whereas large mussels differed in the between-zone transplant, with the mid-zone, non-transplanted mussels upregulating *hsp24* by 4.19 ± 0.16-fold and the transplanted low-zone mussels downregulating expression 0.25 fold (**Figure 3.14**).

Table 3.11 Three-way ANOVA testing the effects of site(region), zone and size on *hsp24* gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	df	MS	F	*p*-value
Zone (mid vs. low)	**0.37**	**1**	**0.37**	**8.60**	**0.0125**
Size (large vs. small)	**1.35**	**1**	**1.35**	**31.43**	**0.0001**
Region (east vs. west coast)	**2.99**	**1**	**2.99**	**69.88**	**<0.0001**
Zone × size	**0.45**	**1**	**0.45**	**10.50**	**0.0071**
Site(region)	**24.22**	**2**	**12.11**	**282.55**	**<0.0001**
Region × zone	0.00	1	0.00	0.02	0.8790
Region × size	**4.93**	**1**	**4.93**	**114.93**	**<0.0001**
Site(region) × zone	0.04	2	0.02	0.49	0.6242
Site(region) × size	**5.53**	**2**	**2.76**	**64.51**	**<0.0001**
Region × zone × size	0.19	1	0.19	4.34	0.0594
Site(region) × zone × size	**0.55**	**2**	**0.28**	**6.45**	**0.0125**
Residuals	0.51	12	0.04		

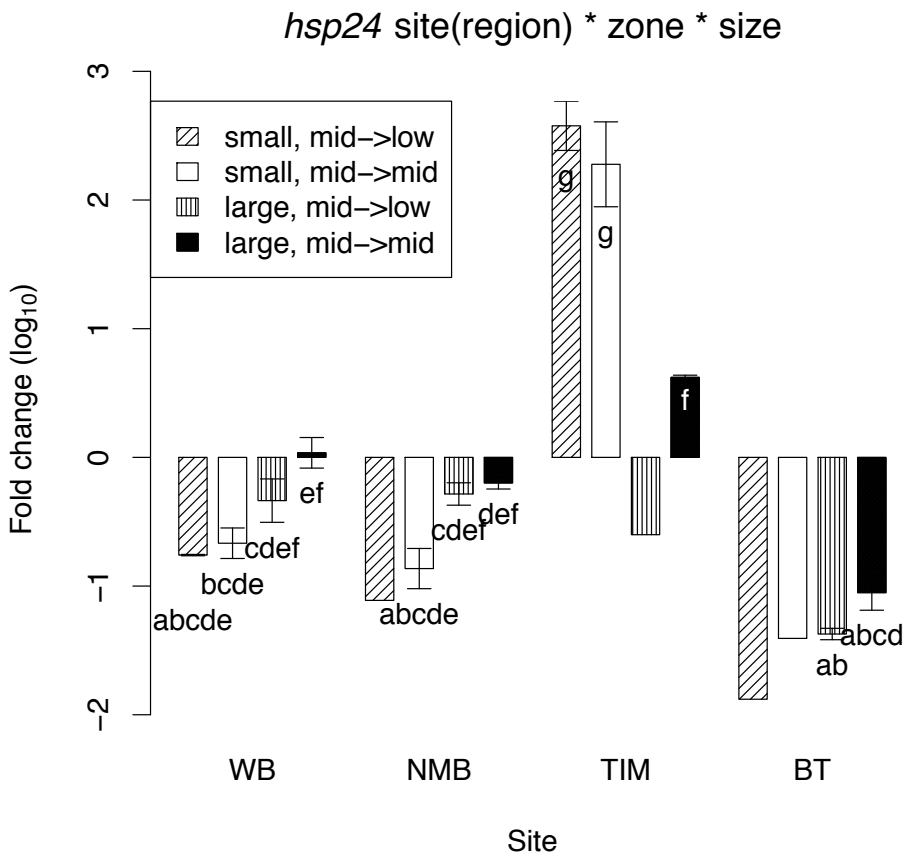


Figure 3.14 *M. galloprovincialis* fold change of *hsp24* gene expression, three-way interaction. Legend format: mussel size, originating zone → translocated zone. Matching letters represent non-significant difference ($F_{2, 12} = 6.447$, $p < 0.05$, Tukey HSD $p < 0.05$).

The small and large mussels at TIM differed significantly in their *hsp24* gene expression ($F_{2, 2} = 64.51$, $p < 0.0001$, Tukey HSD, $p < 0.05$). In the size by site within region comparison, there was very strong *hsp24* upregulation in small mussels at the site TIM (330.61 ± 103.89-fold change; **Figure 3.15**). There was only weak upregulation in large mussels (2.88 ± 1.31–fold change). In general, expression of *hsp24* was enhanced in the small mussels relative to the large mussels. This trend was similar between all four sites despite not being significant at WB. Also, the strongest downregulation occurred at BT, the site with lowest overall aerial temperature, which indicates that temperature may be having an effect on *hsp24* gene expression.

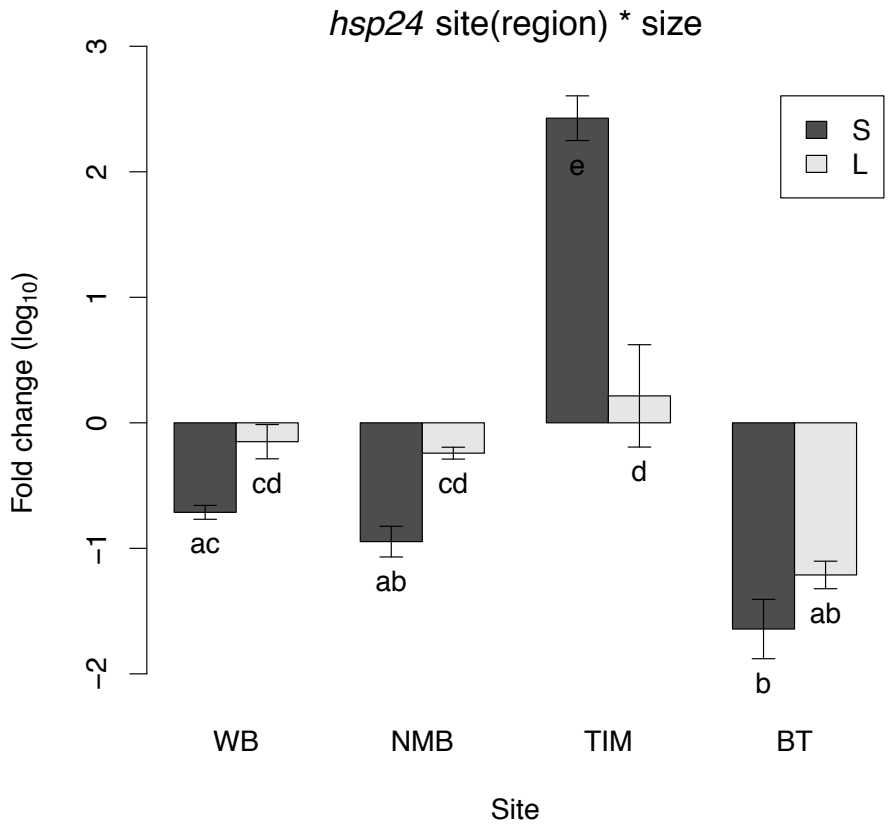


Figure 3.15 *M. galloprovincialis* fold change of *hsp24* gene expression, two-way interaction. S: small mussels, L: large mussels. Matching letters represent non-significant difference ($F_{2, 12} = 64.509$, $p < 0.001$, Tukey HSD $p < 0.05$).

hsp70

Hsp70 expression in *M. galloprovincialis* was downregulated at all sites ($F_{2, 12} = 22.934$, $p < 0.001$, Table 4.5, **Figure 3.16**), but was much lower at TIM (-1.9 ± 0.07) than at the other sites (WB: -0.34 ± 0.06; NMB: -0.25 ± 0.11; BT: -0.54 ± 0.05). Expression at WB, NMB, and BT did not differ (**Table 3.12**, **Figure 3.16**). *Hsp70* expression is generally associated with stress levels, where higher stress elicits an upregulation (Somero, 2011). In this case, expression of *hsp70* at TIM, which is considered to be the most stressful site, is strongly downregulated. HSP70 can inhibit programmed cell death (apoptosis) (Beere, 2004) and cell death can be induced by downregulation of hsp70 (Frese *et al.*, 2003). The downregulation of *hsp70* here suggests that mussels may be close to death at the TIM site. The other three sites are downregulated only slightly, which suggests the stress levels were low.

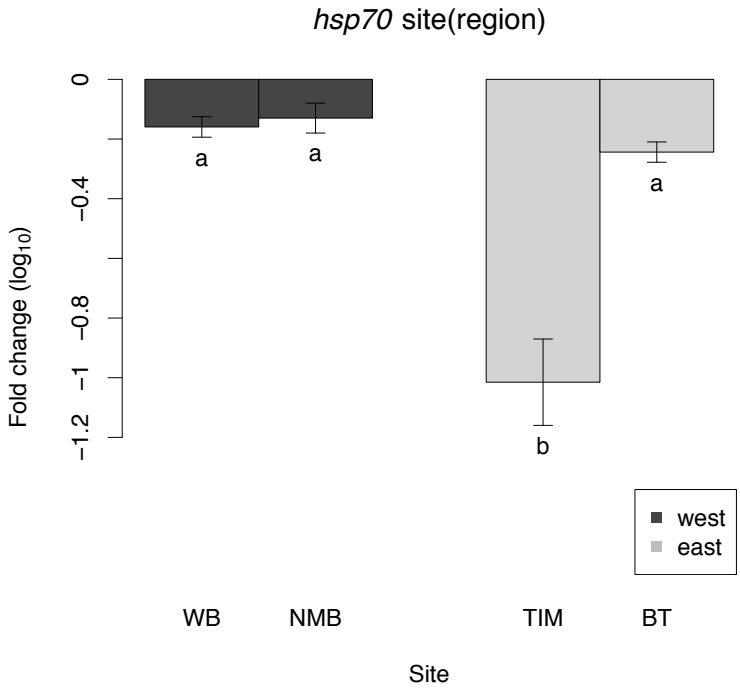


Figure 3.16 *M. galloprovincialis* fold change of *hsp70* gene expression. East: east coast sites (TIM and BT), west; west coast sites (WB, NMB). Matching letters represent non-significant difference ($F_{2, 12} = 22.934$, $p < 0.001$, Tukey HSD $p < 0.05$).

Table 3.12 Three-way ANOVA testing the effects of site(region), zone and size on *hsp70* gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	**df**	**MS**	**F**	***p*-value**
Zone (mid vs. low)	0.08	1	0.08	1.91	0.1920
Size (large vs. small)	0.00	1	0.00	0.06	0.8180
Region (east vs. west coast)	**1.84**	**1**	**1.84**	**43.28**	**<0.0001**
Zone × size	0.09	1	0.09	2.23	0.1610
Site(region)	**1.95**	**2**	**0.98**	**22.93**	**<0.0001**
Region × zone	0.08	1	0.08	1.94	0.1900
Region × size	0.02	1	0.02	0.44	0.5220
Site(region) × zone	0.14	2	0.07	1.61	0.2400
Site(region) × Size	0.05	2	0.03	0.62	0.5550
Region × zone × size ×	0.03	1	0.03	0.82	0.3840
Site(region) × zone × size	0.04	2	0.02	0.49	0.6230
Residuals	0.51	12	0.04		

hsp90

Expression of *hsp90* in *M. galloprovincialis* was markedly upregulated in both size classes at TIM ($F_{2, 8}$ = 26.24, p = 0.0003; large, 4.16 ± 0.41-fold; small, 59.12 ± 6.28-fold; **Table 3.13**; **Figure 3.17**). Small mussels at TIM had higher upregulation of *hsp90* relative to large mussels at TIM and to all other sites. Expression was slightly upregulated at NMB and downregulated at BT, significantly so in large BT mussels.

Table 3.13 Three-way ANOVA testing the effects of site, zone and size on *hsp90* gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	**df**	**MS**	**F**	***p*-value**
Zone (mid vs. low)	0.01	1	0.01	0.67	0.4382
Size (large vs. small)	**3.23**	**1**	**3.23**	**174.74**	**<0.0001**
Site	**7.40**	**2**	**3.70**	**200.03**	**<0.0001**
Zone × size	0.01	1	0.01	0.66	0.4391
Site × zone	0.09	2	0.05	2.43	0.1501
Site × size	**0.97**	**2**	**0.49**	**26.24**	**0.0003**
Site × zone × size	0.02	2	0.01	0.62	0.5623
Residuals	0.15	8	0.02		

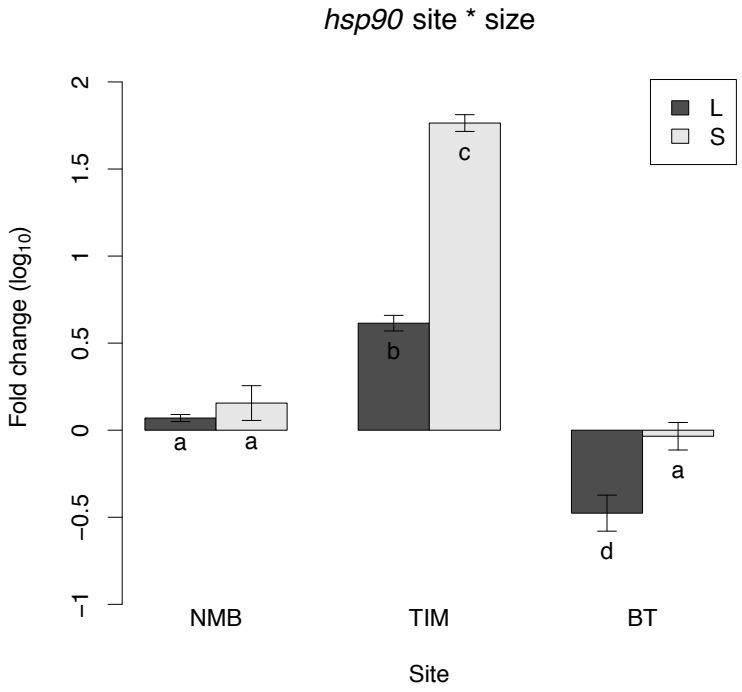


Figure 3.17 *M. galloprovincialis* fold change of *hsp90* gene expression. S: small mussels, L: large mussels. Matching letters represent non-significant difference ($F_{2, 8}$ = 26.24, p = 0.0003, Tukey HSD $p < 0.05$). The WB site is not shown because RT-qPCR reactions failed.

elf2

Small mussels exhibited higher expression (2.08 ± 0.24-fold) than large mussels (1.31 ± 0.27-fold) across all sites and treatments ($F_{1, 6}$ = 11.44, p = 0.0148; **Table 3.14**; **Figure 3.18A**). Overall gene expression of *elf2* in *M. galloprovincialis* at TIM was downregulated, while NMB and BT were upregulated ($F_{2, 6}$ = 23.57, p = 0.0014; **Figure 3.18B**).

Table 3.14 Three-way ANOVA testing the effects of site, zone and size on *elf2* gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	**df**	**MS**	**F**	***p*-value**
Zone (mid vs. low)	0.00	1	0.00	0.08	0.7936
Size (large vs. small)	**0.39**	**1**	**0.39**	**11.44**	**0.0148**
Site	**1.59**	**2**	**0.80**	**23.57**	**0.0014**
Zone × size	0.00	1	0.00	0.02	0.8939
Site × zone	0.04	2	0.02	0.54	0.6103
Site × size	0.00	1	0.00	0.02	0.8988
Site × zone × size	0.00	1	0.00	0.03	0.8764
Residuals	0.20	6	0.03		

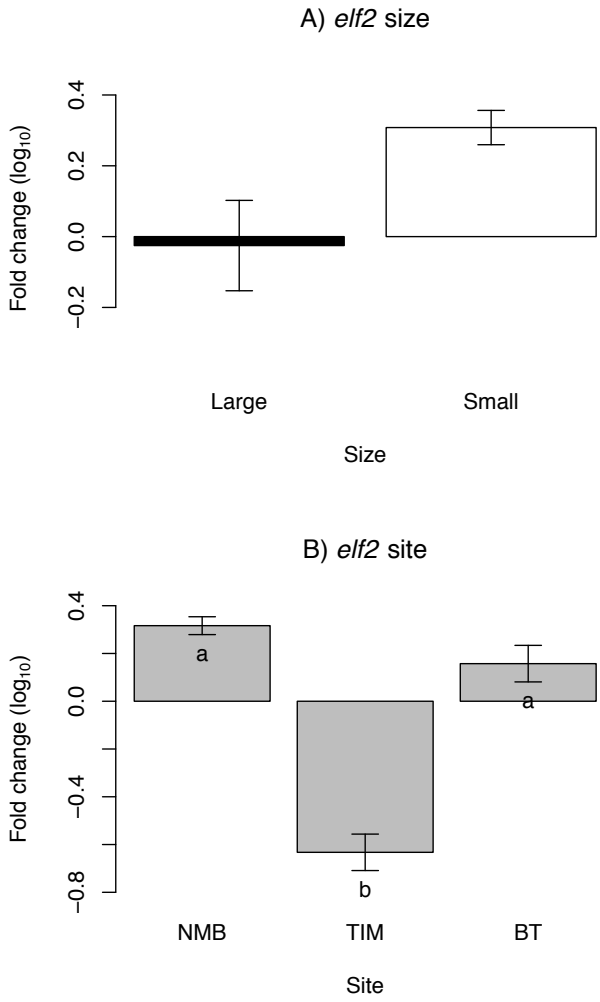


Figure 3.18 *M. galloprovincialis* fold change of *elf2* gene expression. A) size ($F_{1, 6}$ = 11.44, p = 0.0148, Tukey HSD $p < 0.05$), B) site ($F_{2, 6}$ = 23.57, p = 0.0014, Tukey HSD $p < 0.05$). Matching letters represent non-significant difference.

tis11d

Strong downregulation of *tis11d* was seen at the sites WB and TIM ($F_{2, 12}$ = 6.062, $p < 0.05$; **Table 3.15**; **Figure 3.19**). However, at TIM it was small mussels and at WB large ones whose expression was downregulated. The strongest upregulation was seen in small mid-zone mussels at NMB ($F_{2, 12}$ = 30.684, $p < 0.001$; **Figure 3.20**).

Table 3.15 Three-way ANOVA testing the effects of site(region), zone and size on *tis11d* gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	**df**	**MS**	**F**	***p*-value**
Zone (mid vs. low)	0.05	1	0.05	2.52	0.1385
Size (large vs. small)	**0.23**	**1**	**0.23**	**11.89**	**0.0048**
Region (east vs. west coast)	**0.43**	**1**	**0.43**	**21.67**	**0.0006**
Zone × size	0.01	1	0.01	0.70	0.4196
Site(region)	**1.78**	**2**	**0.89**	**45.26**	**<0.0001**
Region × zone	0.02	1	0.02	0.83	0.3797
Region × size	**1.69**	**1**	**1.69**	**86.14**	**<0.0001**
Site(region) × zone	0.02	2	0.01	0.62	0.5571
Site(region) × size	**1.20**	**2**	**0.60**	**30.68**	**<0.0001**
Region × zone × size	0.00	1	0.00	0.01	0.9375
Site(region) × zone × size	**0.24**	**2**	**0.12**	**6.06**	**0.0151**
Residuals	0.24	12	0.02		

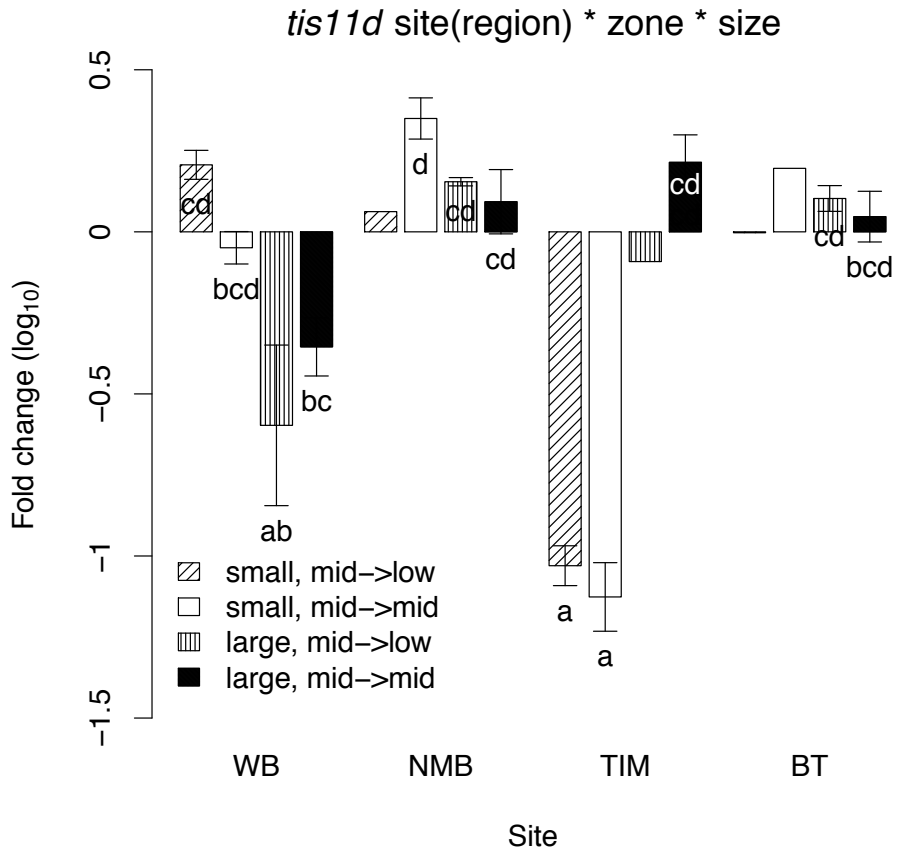


Figure 3.19 *M. galloprovincialis* fold change of *tis11d* gene expression, three-way interaction. Legend format: mussel size, originating zone → translocated zone. Matching letters represent non-significant difference ($F_{2, 12} = 6.062$, $p < 0.05$, Tukey HSD $p < 0.05$).

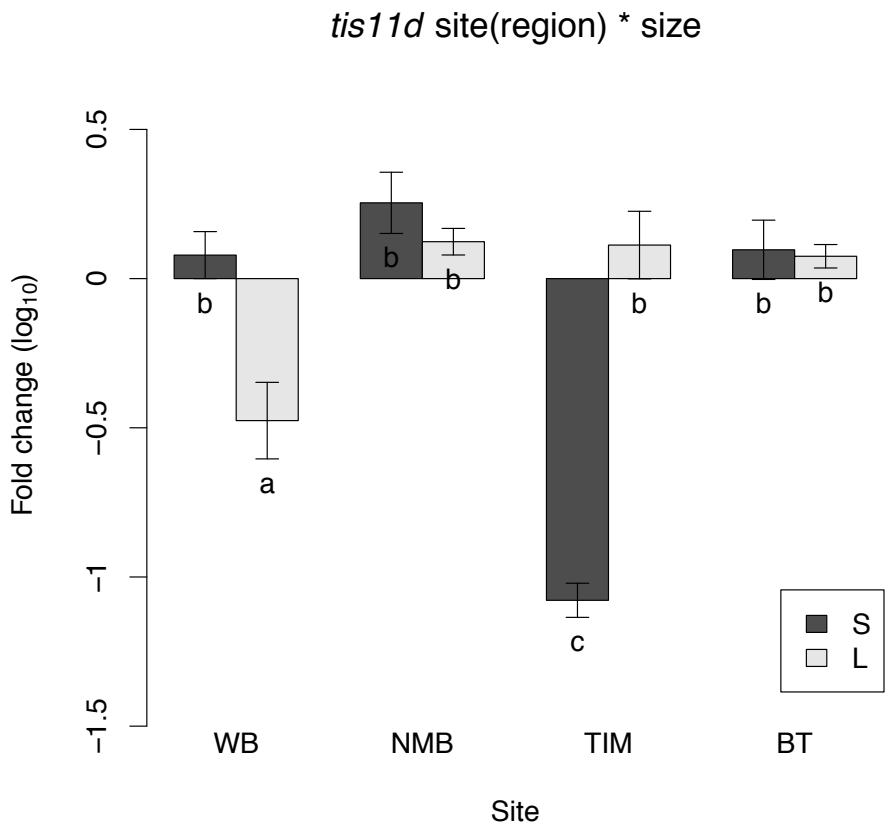


Figure 3.20 *M. galloprovincialis* fold change of *tis11d* gene expression, two-way interaction. S: small mussels, L: large mussels. Matching letters represent non-significant difference ($F_{2, 12} = 30.684$, $p < 0.001$, Tukey HSD $p < 0.05$).

hsp70 P. canaliculus

Once again, it was the TIM site that showed strongest upregulation, this time for small *P. canaliculus* (72.43 ± 26.20-fold; $F_{2, 7} = 59.082$, $p < 0.001$; **Table 3.16**; **Figure 3.21**). Members of this size class upregulated *hsp70* at WB whereas they downregulated expression at the other three sites. There was a significant between-zone interaction in the GLM ($F_{2, 7} = 6.47$, $p < 0.03$), however the Tukey's HSD ($p < 0.05$) test shows that there the expression of *hsp70* was consistent between zones at each site and the only differences between zones were across different sites.

Table 3.16 Three-way ANOVA testing the effects of site(region), zone and size on *hsp70* gene expression in *P. canaliculus*. Test factors in bold were significant ($p < 0.05$).

	SS	df	MS	F	*p*-value
Zone (mid vs. low)	0.01	1	0.01	0.71	0.4282
Size (large vs. small)	**1.99**	**1**	**1.99**	**150.95**	**<0.0001**
Region (east vs. west coast)	0.05	1	0.05	3.76	0.0938
Zone × size	**0.30**	**1**	**0.30**	**22.49**	**0.0021**
Site(region)	**3.22**	**2**	**1.61**	**122.33**	**<0.0001**
Region × zone	0.03	1	0.03	1.98	0.2018
Region × size	**1.52**	**1**	**1.52**	**115.04**	**<0.0001**
Site(region) × zone	**0.17**	**2**	**0.09**	**6.47**	**0.0257**
Site(region) × size	**1.56**	**2**	**0.78**	**59.08**	**<0.0001**
Region × zone × size	0.00	1	0.00	0.12	0.7350
Site(region) × zone × size	0.00	1	0.00	0.27	0.6225
Residuals	0.09	7	0.01		

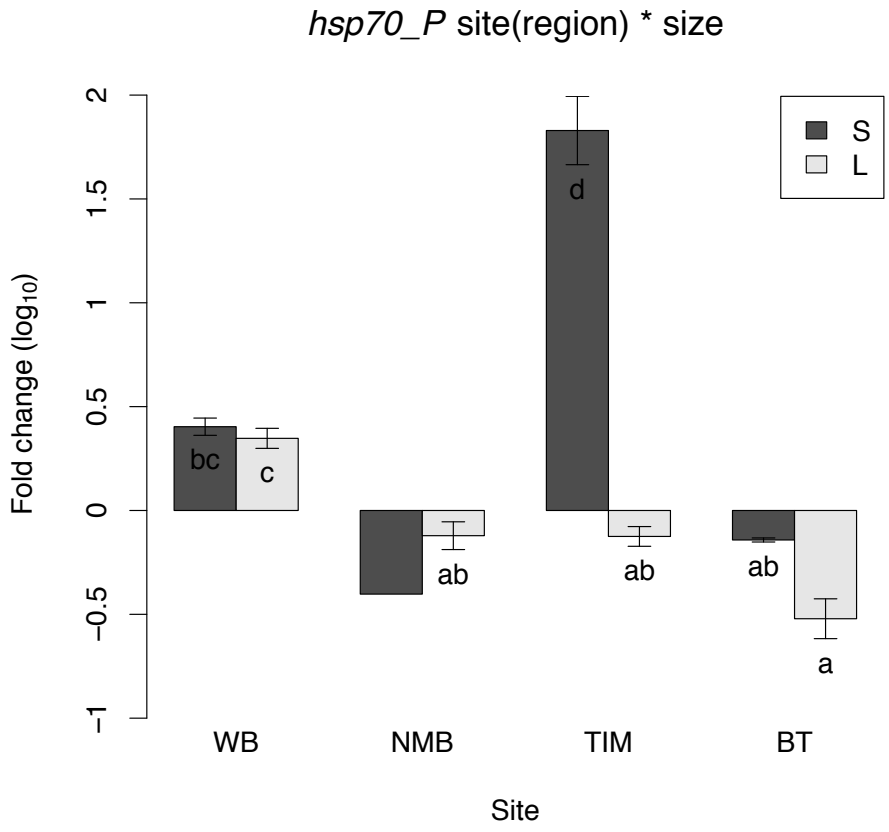


Figure 3.21 *P. canaliculus* fold change of *hsp70* gene expression. S: small mussels, L: large mussels. Matching letters represent non-significant difference ($F_{2, 7} = 59.082$, $p < 0.001$, Tukey HSD $p < 0.05$).

3.3.8 Results summary

In the *M. galloprovincialis* multivariate analysis, there were several significant factors (**Table 3.17**). Most of the variability is found on the x-axis of the multivariate analysis, which is most strongly associated with the expression of *hsp24*, and average daily maximum temperature (**Figure 3.22**). The TIM site was distinct from the rest of the sites. Here, it was the combination of extreme temperatures with longer emersion times that differed greatly from the other sites. Any differences of 'coasts', therefore, were largely driven by this site. Of the predictor variables (region, site, zone, size), site was the only significant factor (**Table 3.17**). Average daily maximum temperature was also significant. Ultimately, for *M. galloprovincialis,* the main source of variability in growth, survival and gene expression was from the site occupied by the mussels.

Table 3.17 Tests for relationships between gene expression and biological and physical variables in *M. galloprovincialis* using non-parametric multivariate regression analysis. Temperature is the average daily maximum air temperature, emersion is the hours per day air exposure, and TSI is the sum of temperature during air exposure. Test factors in bold were significant ($p < 0.05$).

	R^2	*p*-value
Explanatory variables		
Temperature	**0.36**	**0.0076**
Emersion	0.07	0.4258
TSI	0.09	0.3417
region	0.09	0.1383
size	0.04	0.3193
site	**0.69**	**0.0001**
zone	0.001	0.9508
Response variables		
survival	0.24	0.0524
growth	0.12	0.2286
hsp70	**0.93**	**0.0001**
tis11d	**0.78**	**0.0001**
hsp24	**0.99**	**0.0001**

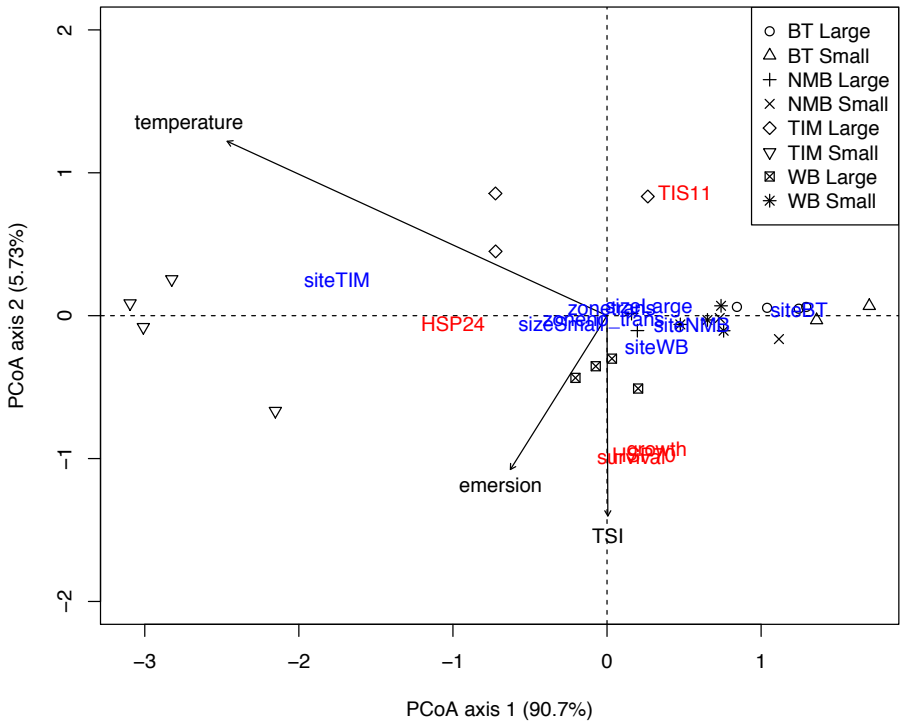


Figure 3.22 Principle coordinates analysis of *M. galloprovincialis* survival, growth and gene expression (*hsp24, hsp70* and *tis11d)* (red). Temperature is the average daily maximum air temperature, emersion is the hours per day air exposure, and TSI is the sum of temperature during air exposure. Temperature and site were significant factors ($p < 0.05$).

In the *P. canaliculus* multivariate analysis, temperature ($p = 0.0074$) and size ($p = 0.0002$) were significant factors for growth and survival (**Table 3.18**; **Figure 3.23**). Once again, the TIM site stands out.

Table 3.18 Tests for relationships between gene expression and biological and physical variables in *P. canaliculus* using non-parametric multivariate regression analysis. Temperature is the average daily maximum air temperature, emersion is the hours per day air exposure, and TSI is the sum of temperature during air exposure. Test factors in bold were significant ($p < 0.05$).

	R^2	*p*-value
Explanatory variables		
Temperature	**0.44**	**0.0074**
Emersion	0.27	0.0647
TSI	0.29	0.0521
region	0.1	0.1859
size	**0.53**	**0.0002**
site	0.18	0.3592
zone	0.13	0.126
Response variables		
survival	**1**	**0.0001**
growth	**1**	**0.0001**
hsp70_P	0.23	0.0996

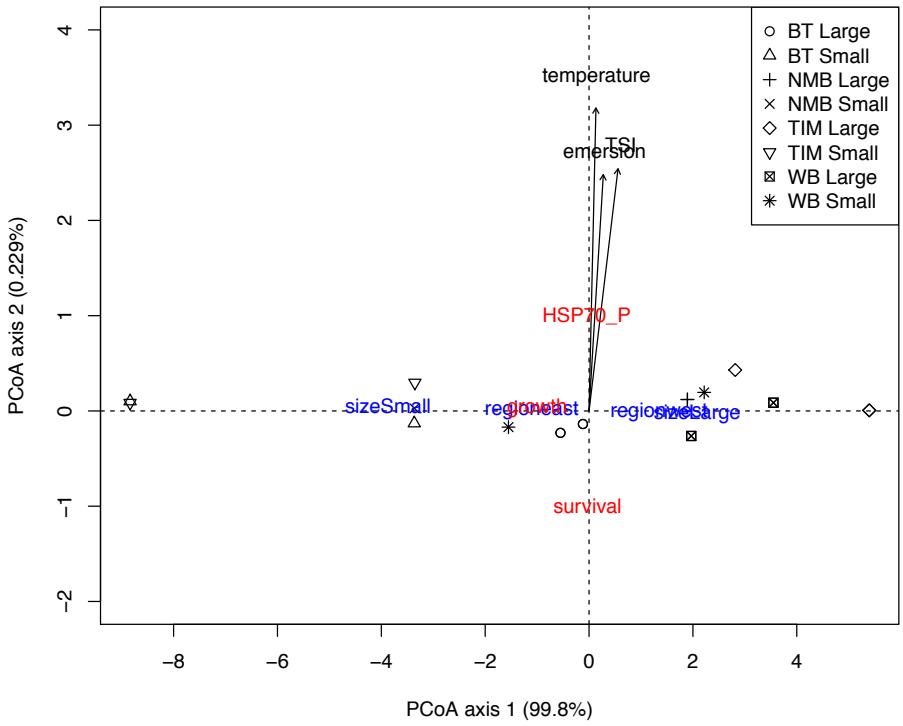


Figure 3.23 Principle coordinates analysis of *P. canaliculus* survival, growth and *hsp70* gene expression (red). Temperature is the average daily maximum air temperature, emersion is the hours per day air exposure, and TSI is the sum of temperature during air exposure. Size and temperature were significant factors ($p < 0.05$).

3.4 Discussion

The major effects seen in this experiment were the result of site-specific characteristics. Growth and survival were measured along with gene expression to determine if there were differences in stress response between species, size classes, intertidal zones or sites. In particular, the Timaru site experienced very hot temperatures and long emersion (air exposure) times, which combined to reduce overall survival rates and affect gene expression. Site had a strong impact on stress response, much stronger than intertidal zone, mussel size or species-specific differences. Gene expression at the TIM site was markedly different than expression at the other sites for all the stress response genes studied here. Few differences were found between species or zone, which was surprising given that the species naturally occupy different intertidal zones and *P. canaliculus* is known to be less tolerant to stressful conditions than *M. galloprovincialis* (Morton, 2004; Menge *et al.*, 2007; Petes *et al.*, 2007)

Challenging environmental conditions intensify with increasing tidal elevation. *P. canaliculus* tends to live in the low intertidal zone and *M. galloprovincialis* is more abundant in the more challenging mid intertidal zone (Morton, 2004). The boundary between these two species is not abrupt and there is a region where they co-occur (Menge *et al.*, 2007). In this region, Petes *et al.* (2007) showed that *P. canaliculus* are less tolerant than *M. galloprovincialis* to extreme heat events (Petes *et al.*, 2007). In my study, comparisons between species could be directly made for growth, survival and *hsp70* gene expression. The results indicate that *P. canaliculus* was less affected by environmental stress than *M. galloprovincialis*, which runs counter to predictions based on the natural zonation patterns of the two species and the results from Petes *et al. (2007)*. *M. galloprovincialis* survival was worse than *P. canaliculus*, growth was slightly lower in small mussels and *hsp70* expression was strongly repressed at the most challenging site (TIM). The intense downregulation of *hsp70* at TIM suggests that *M. galloprovincialis* may have been near death (Frese *et al.*, 2003). Expression of *hsp70* has been linked with cell death (apoptosis) where high *hsp70* blocks apoptosis (Feder and Hofmann, 1999; Gracey *et al.*, 2008) and downregulation of *hsp70* induces apoptosis (Frese *et al.*, 2003). *P. canaliculus* was also stressed at TIM, with high mortality, upregulated *hsp70* expression and low growth. However, the physiological response to stress by *P. canaliculus* was less pronounced than that of *M. galloprovincialis*. The cause for the enhanced response to stress by *M. galloprovincialis* is unclear and further study is required to better understand differences in the stress response mechanisms of the two species in harsh environments like TIM.

Small mussels of both species were more sensitive to physiological stress than large mussels. All but one of the genes tested showed stronger changes in expression in small mussels compared to large mussels. *Hsp70* expression in *M. galloprovincialis* was not significantly different between the two size classes. This result followed the predicted hypothesis that the effects of stress would be more substantial for smaller mussels. Mussels grow at different rates at different ages throughout their lifespan. Young (small) mussels grow more quickly than older (large) mussels (Sukhotin *et al.*, 2002) (**Figure 3.13C**). The rate of oxidative metabolism is also greater in younger mussels (Sukhotin *et al.*, 2002), which could lead to increased metabolic by-products. The ability to protect the cells from

environmentally-induced damage may be compromised if animals must expend energy to prevent oxidative damage from metabolism. Additionally, large animals have a larger surface area to body mass ratio than small individuals, therefore their capacity to withstand stressful conditions may be higher (Pellegrino, 1984).

The experiment was done in two regions, west and east coasts, with site replication in each region. Extensive water sampling has been carried out by Menge and colleagues for nearly 20 years (Menge *et al.*, 1999; Menge *et al.*, 2002; Menge *et al.*, 2003; Bracken *et al.*, 2012) and has consistently shown that nutrients off the west coast are substantially higher than the east coast. The origin of nutrient input is debated, but Menge (1999) suggests it results from upwelling along the west coast of the South Island although there may also land-derived sources via freshwater run-off (Schiel, 2004). Three of the sites in the present study (BT, WB and NMB) are long-term study sites of Menge. To my knowledge, TIM has not been previously used as an experimental field site, and there are no long-term data on nutrients, recruitment or ecological processes at TIM. However, due to patterns of ocean circulation around the South Island, nutrients are likely to be low (Knox, 1963; Mann and Jones, 2003). The amount of food available to intertidal organisms can impact how severe the stress response is to difficult environmental conditions (Dahlhoff and Menge, 1996; Norkko *et al.*, 2005; Schneider *et al.*, 2010; Núñez-Acuña *et al.*, 2012). In my studies, the animals had the highest stress response at TIM, a site with suspected low food.

Strong negative effects on the survival and physiology of mussels occur when thermal stress is combined with low food or hypoxia (Anestis *et al.*, 2010a; Schneider *et al.*, 2010). For example, thermal stress in conjunction with limited food has a strong negative effect on survival in *M. galloprovincialis* and *M. trossulus* from the US west coast (Schneider *et al.*, 2010). The two species were subject to simulated tidal regimes and three temperature treatments (20, 25, 30˚C) in high and low food conditions. Survival in the hot treatment dropped for *M. galloprovincialis* and *M. trossulus* from 57% to 33% and 38% to 16% respectively when food was limited (Schneider *et al.*, 2010). Given that global thermal conditions are increasing and net primary productivity is decreasing (Behrenfeld *et al.*, 2006), interactive effects of these high temperature and low food conditions could be deleterious for mussel populations (Schneider *et al.*, 2010). In my study, similar factors may be at play at the TIM site with extreme temperatures and

suspected lower food on the east coast possibly driving the upregulated gene expression, low growth stress responses in *P. canaliculus* and *M. galloprovincialis*.

In addition to the combination of food and temperature, low tide-induced hypoxia has also been shown to compound the effects of stressful conditions (Anestis *et al.*, 2010a). Metabolic by-products accumulate when aquatic organisms are emersed since water is necessary for gas exchange and excretion (Bayne *et al.*, 1976; Helmuth *et al.*, 2005). Mussels can alter their metabolic rate to cope with low-tide-induced hypoxia (Bayne *et al.*, 1976; Connor and Gracey, 2011) and the heat shock response can be initiated in times of hypoxic stress, especially when combined with high temperatures (Anestis *et al.*, 2010a) or low food (Norkko *et al.*, 2005). For example, there was no mortality for up to 48h when *M. galloprovincialis* were exposed to 18˚C air temperature after they had been acclimated to 18˚C (Anestis *et al.*, 2010a). However, when those mussels were exposed to 32˚C air temperatures, 15% of mussels died within 10h and 53% died within the next 10h of exposure. The hypoxic environment of air exposure was exacerbated by high temperature and induced metabolic (key glycolytic enzyme pyruvate kinase) and molecular (Hsp70 and Hsp90 expression, and phosphorylation of stress-activated protein kinases p38 MAPK and cJun-N-terminal kinase) stress responses (Anestis *et al.*, 2010a). In my study, the combination of particularly long emersion times at coupled with extreme temperatures, primarily at TIM, are likely to have influenced the upregulation of stress response gene expression and compromised growth and survival. As environmental conditions change due to global climate change driven processes, intertidal animals may be vulnerable due to the interactive effects of temperature and other abiotic factors.

The rocky intertidal is an inherently stressful place to live, with organisms having to cope with both aquatic and aerial conditions on a daily basis. The effects of challenging abiotic conditions are stronger in the mid to high intertidal zone than in the low-zone because the emersion time is greater. However, there were few between-zone differences in stress response for either *M. galloprovincialis* or *P. canaliculus* in this study. Extreme temperatures or other stressful factors (such as hypoxia or nutrient levels) may have overwhelmed the differences in stress between intertidal zones. *P. canaliculus* is normally found in the low intertidal zone (Menge *et al.*, 1999; Morton, 2004; Menge *et al.*, 2007) but had limited mid-zone stress responses: survival was not different between zones, and growth was

only different between zones at the TIM site and there was no difference between zone for *hsp70* expression. There were also few between zone differences for *M. galloprovincialis*. The absence of between-zone stress response at BT could result from the similarity of abiotic conditions, where there was little temperature difference between zones. However, the absence of between-zone stress response at TIM could result from extreme temperatures and high emersion times in both intertidal zones overwhelming the other abiotic differences that are different between intertidal zones. Temperature is a primary driver of biogeographic distribution for intertidal organisms (Hochachka and Somero, 2002). Therefore if overall thermal stress was extremely high then other stressors such as desiccation or hypoxia that vary between zones may not have greatly influenced the organisms.

Predation is often an important factor for survival and stress but it is unlikely to have had a large effect in these experiments. Mobile invertebrate predators (whelks and sea stars) on the east coast of New Zealand are rare (Menge *et al.*, 1999; Menge *et al.*, 2002). There is strong fish and crab predation but it is limited to small mussels (< 15 mm in size; Rilov and Schiel, 2006b; a). Sea stars and whelks are abundant and predation is high on the west coast, but only in the low-zone (Menge *et al.*, 1999; Menge *et al.*, 2002). The only between-zone differences in survival on the west coast in these experiments were with small mussels (20-40 mm) at NMB, but this mortality is probably the result of the mussels being smothered by sand rather than predation (**Figure 3.11**).

Stress could also be caused by inter- or intraspecific competition (Menge and Sutherland, 1987). The plots in this study only contained a single species, so interspecific competition was not a factor. The experiment began with 25 mussels per plot. Since the mussels were not in a dense mussel bed competing for space, it is unlikely that intraspecific competition played a role in stress response.

The small patch size of mussel clumps used in this study could have influenced the stress response. Environmental buffering from the mussel bed is limited in small patches of mussels (Cole, 2010). However, mussels frequently appear in small clumps in natural populations, so this is unlikely to have been a major driver of stress response. Furthermore it is unlikely to have driven the stress response at only one particular site (TIM) where stress response was highest.

In planning qPCR gene expression studies, the first task is to select candidate genes. Previous studies have shown that the genes selected for use here respond to stress (Place *et al.*, 2008; Lockwood *et al.*, 2010). The genes selected were used for markers of stress to address how different species and size classes of mussel response to stressful environmental conditions. Detail about the specific mechanism of stress is not provided by the genes used here. Other genes would be required to investigate the mechanisms of stress. For example, apoptosis markers such as P53 (Kiss, 2010) or catalase, which responds specifically to oxidative stress (Greco *et al.*, 2011) could be used to investigate physiological mechanisms of stress. There are few annotated sequences for molluscs (Sadamoto *et al.*, 2012) and to identify *de novo* genes that would be expected to respond to specific stressors would be a nearly impossible task. The use of next generation sequencing (NGS) technology has potential to overcome this difficulty. NGS enables gene discovery and can be used on organisms that do not have large genomic database resources (Sadamoto *et al.*, 2012).

Some difficulties inherent with field experiments are all the conditions that cannot be controlled. Ecologists can overcome the (sometimes extreme) natural variability and extract meaningful results from field experiments with adequate sample sizes. However, the high cost of analyses becomes a critical factor in experimental design when molecular analyses are coupled with ecological studies. Care must be taken to ensure adequate sample sizes in the field while maintaining a reasonable cost for the project overall. In this study, there was high variability in several areas (temperature between the different field sites, magnitude of gene expression within single treatments, and survival), which can lead to difficulty in the interpretation of results. More sites and larger sample sizes should be used for field-based stress responses studies if this were to be repeated in the future.

The potential for genetic variability between the different populations must be considered when using multiple sites (Oleksiak *et al.*, 2002). Genetic structure of *P. canaliculus* populations around the South Island of New Zealand are more diverse than around the North Island (Apte and Gardner, 2002). The few studies available focus primarily on mussels from the North Island (*P. canaliculus* or *M. galloprovincialis*) and do not thoroughly investigate the population structure around the South Island (Apte and Gardner, 2002; Star *et al.*, 2003; Wood *et al.*, 2007; Westfall *et al.*, 2010; Gardner and Westfall, 2012). There is a consistent break

in genetic population structure between the North and South Islands of New Zealand. There is some evidence that shows *P. canaliculus* at TIM are genetically similar to mussels from the North Island despite having a South Island specific haplotype (Apte and Gardner, 2002). The unusual genetic makeup of mussels from TIM indicates that the stress response seen there may have been influenced by the genetic makeup of the population. Given that little is known about the population structure of mussels around the South Island of New Zealand, future analyses of this sort would be beneficial to better understand the connectivity of populations and whether differences in stress response were affected by the underlying genetic population structure among populations or directly by the environmental conditions.

In hindsight, this study could have been stronger by considering some additional factors. Food availability should have been measured directly during the experiments rather than relying on the historical data from Menge's work (Menge *et al.*, 1999; Menge *et al.*, 2003; Menge *et al.*, 2007; Bracken *et al.*, 2012). Four sites were not enough to draw strong conclusions about the physiological responses seen in the mussels of this study due to the lack of striking results. It was anticipated that there would have been larger changes in gene expression than was seen based on work from Place *et al.* (2008) and Lockwood *et al.* (2010).

Comparisons between coasts were lost for several of the analyses due to the loss of a temperature data logger in the low-zone at Nine Mile Beach. Unfortunately, this had a large effect on the analyses that could be carried out. In future, I would be recommend that multiple data loggers are used for each treatment to provide redundancy in the experimental design.

Investigation of gene expression within and among populations is not frequently undertaken due to the difficulty of teasing apart the results from genetic variability between populations and within populations. Inter-individual variation in gene expression has been studied at length by Crawford and Oleksiak (Oleksiak *et al.*, 2002; Whitehead and Crawford, 2006; Crawford and Oleksiak, 2007). They have shown that many small changes in different suites of genes can lead to changes in cardiac metabolism (Crawford and Oleksiak, 2007). However, it is not necessarily the same genes that change their expression, but rather small changes in different genes to end up with the same outcome (Crawford and

Oleksiak, 2007). In the mussels studies here, there was a large amount of variation in the gene expression results which may have been due to individual variability.

This study set out to investigate differences in stress response between two species of intertidal mussel, *M. galloprovincialis* and *P. canaliculus.* The effects of stress on different size classes (large and small) and in different intertidal zones (mid and low) at different sites around New Zealand were assessed. Some, but not all of the hypotheses I set out to test in this study were supported. (1) There were no between-zone difference in stress response, contrary to predictions; (2) Small mussel did respond more to stress than large mussels, as expected; (3) *P. canaliculus* performed better than *M. galloprovincialis,* counter to expectations; (4) There was a higher stress response on the east coast, but only for one site, TIM. Further investigation is needed to fully understand the physiological effects of environmental stress associated with predicted climate changes. Many comparisons showed no significant differences between treatments. This could be overcome by larger sample sizes, and more study sites to reduce variability. Subsequent investigations into the detailed mechanisms of stress should be undertaken which is made easier by the development of NGS. For example, transcriptome analyses could provide detailed information stress induced expression patterns. This would increase the understanding of the mechanistic effects of stress on mussel physiology and how climate change-related environmental effects may influence rocky intertidal communities.

Chapter Four:

Adaptive responses to variable environments

4.1 Introduction

In response to variable and changing environmental conditions, the extent of an organism's ability to respond effectively physiologically is crucial to survival. This is particularly important where environmental conditions can be extreme and fluctuate dramatically, such as in the intertidal zone. Organisms that live in such habitats, like the marine intertidal zone, can exist near their physiological maxima and may have limited ability to adapt to more extreme conditions due to the nature of cellular response mechanisms (Stenseng *et al.*, 2005; Somero, 2011). Animals that live in extremely stable environments, such as Antarctic fishes can lack acclimatory responses altogether (Hofmann *et al.*, 2000). Furthermore, animals in moderately variable environments are likely to have more capacity for adaptive change (Somero, 2011). Physiological adaptive change is the maintenance and adaptation of core biochemical processes such as proteins and enzymes, nucleic acids and cell membranes (Hochachka and Somero, 2002). The normal cellular machinery and interactions between cellular components are highly vulnerable to perturbations. Therefore, maintenance of functional properties is required. If the functional balance between cellular components shifts too far into an unstable state due to stress-induced damage, this damage can become beyond repair. If animals cannot adapt physiologically or if cellular damage is too severe, then localised extinction of populations and range shifts may occur (e.g., *Mytilus edulis*, east coast USA (Jones *et al.*, 2010), invertebrate communities in the UK (Southward *et al.*, 1995)).

A frequently studied physiological stress mechanism is the heat shock response, in particular the heat shock proteins (HSPs) (Hochachka and Somero, 2002). HSPs function as molecular chaperone proteins to prevent and repair damaged proteins (Feder and Hofmann, 1999; Podrabsky and Somero, 2004; Tomanek, 2010). Environmental stress can induce damage to proteins by influencing the cellular environment through changes in temperature, salinity or other conditions (Hochachka and Somero, 2002). The intensity of stress responses has been correlated to the magnitude of hsp70 upregulation, and hsp70 expression levels are frequently used as indicators of environmental stress (Feder and Hofmann, 1999).

There is an increasing body of literature showing that different organisms have different abilities to cope with thermal stress. For example, the marine snail

Chlorostoma (formerly *Tegula*) has three congeneric species that live at different tidal elevations and have differing adaptive capabilities in upper lethal temperatures (Stenseng *et al.*, 2005). The lower intertidal zone species (*C. brunnea* and *C. montereyi*) have more acclimatory capacity to increased thermal conditions than the high-zone species (*C. funebralis*) and therefore are at a lower risk to the effects of climate change. Competition for space among other species on the low shore may prevent high-zone organisms from relocating lower on the shore to alleviate stressful conditions (Hawkins *et al.*, 2008). Similar responses have been seen in congeneric species of the porcelain crab *Petrolisthes* (Stillman, 2002).

There can be differences in thermal physiology between different populations of one species. Mussels of the same species (*M. californianus*) collected from seven sites ranging from northern Washington to Baja California maintained population level differences in thermal physiology when exposed to acute thermal stress (Logan *et al.*, 2012). This is despite being acclimated to common conditions before testing. Northern mussels had significantly lower survival than southern mussels after acute heat shock. Also, northern mussels reached a critical heart rate at lower temperatures than mussels from southern sites. Logan *et al.* (2012) hypothesised that the differences may have resulted from local genetic adaptation or phenotypic plasticity that became fixed within a population during development.

Physiological tolerances of marine organisms in response to environmental stress can be experimentally tested using rocky intertidal sites with different environmental conditions because organisms can readily be transplanted between sites. Rocky intertidal sites on the east coast of New Zealand are exposed to varying oceanographic conditions, which result in different nutrient inputs to the intertidal communities (Knox, 1963; Menge *et al.*, 2003). Factors such as food, desiccation and thermal conditions influence the physiological state of animals that occupy the intertidal zone. Therefore, examination of the adaptive responses at different sites over the course of the warmer summer months can provide underpinning information about how intertidal animals adapt to variable or more extreme conditions.

Stress response studies among species over large geographic areas have given mixed results. Some studies show that local conditions determine stress responses (Helmuth, 1998) while several others show latitudinal gradients in

stress response patterns (Sorte and Hofmann, 2004; Osovitz and Hofmann, 2005). Unfortunately, in these latitudinal studies, the results could be influenced by the genetic structure of populations, as suggested by Logan *et al.* (2012). Since the organisms studied originate from different populations, local adaptation to conditions may be influencing the physiological stress response.

To address the issue of population genetic adaptation, one population of *P. canaliculus* was used to measure stress responses to varying environmental conditions through transplantations between sites with different environmental conditions in the present study. *P. canaliculus* was chosen for this study as it was anticipated that the stress response would be greater than that of the mid-shore species *M. galloprovincialis. P. canaliculus* is found predominantly in the low intertidal zone and shallow subtidal zone (Morton, 2004) and is considered to have lower physiological tolerance to environmental stress than *M. galloprovincialis* (Menge *et al.*, 2007; Petes *et al.*, 2007). *Hsp70* gene expression levels, survival and growth were used to assess stress levels of one population of *P. canaliculus*. Stress response was tested across variable environments over the hot summer months when stress would have been greatest. Mussels were transplanted from a source population to locations with different environmental conditions.

The three sites in this experiment differed naturally in the density of mussels. At Cave Rock, the source population, there are dense mussel beds covering much of the exposed rocky surface. Raramai, 170 km to the north, has fewer mussels and grazing invertebrates are prevalent on the shore. Shag Point, 300 km to the south, has few mussels and a larger cover of algae than Raramai and Cave Rock (Menge *et al.*, 2003). Cave Rock is near the output of the Avon-Heathcote estuary, and therefore the nutrients in the water are likely to be high (Environment Canterbury Regional Council, 2010). The areas around Cave Rock have higher levels of mussel recruitment, competition and phytoplankton (chlorophyll-*a* concentrations) relative to Raramai and Shag Point (Menge *et al.*, 2003; Environment Canterbury Regional Council, 2010). Shag Point has much lower particulates than near Cave Rock or Raramai. The air temperatures during low tide near Raramai and Shag Point can reach 35.5˚C and 28.0˚C respectively (Lilley and Schiel, 2006), while near Cave Rock they can reach 26.0˚C (Menge *et al.*, 1999).

The extensive between-site differences in environmental conditions are likely to have an effect on the physiological state of mussels living at those sites. Additional site descriptions are found in **Section 1.5**. Specifically, this chapter tests the hypothesis that survival will be compromised in mussels transplanted from one source population to sites with more challenging environmental conditions. Mussels that survive in these new locations will show evidence of physiological stress such as elevated *hsp70* expression and decreased growth.

4.2 Methods

This study included ecological and molecular components. Field transplants manipulated the location of mussels between three sites spanning four degrees of latitude on the east coast of the South Island in the summer months of December 2009 – April 2010. RT-qPCR was used to examine the gene expression of *hsp70* in *P. canaliculus* in response to these field transplantations.

4.2.1 Study sites

Mussels were shifted from a source population to two other locations with different environmental conditions (**Figure 4.1**). Cave Rock, was the source site and the two transplant sites were Raramai and Shag Point. Cave Rock was chosen as the source site partly due to the biosecurity risk of the invasive seaweed, *Undaria pinnatifida*. *U. pinnatifida* is not yet present at the Raramai or Shag Point sites, but it is present at Box Thumb, which is a site that was used in the experiments from Chapter Four. Since the mussels in this experiment were transplanted to sites where *U. pinnatifida* does not yet occur, Cave Rock was selected as the source site to prevent transmission of the invasive species.

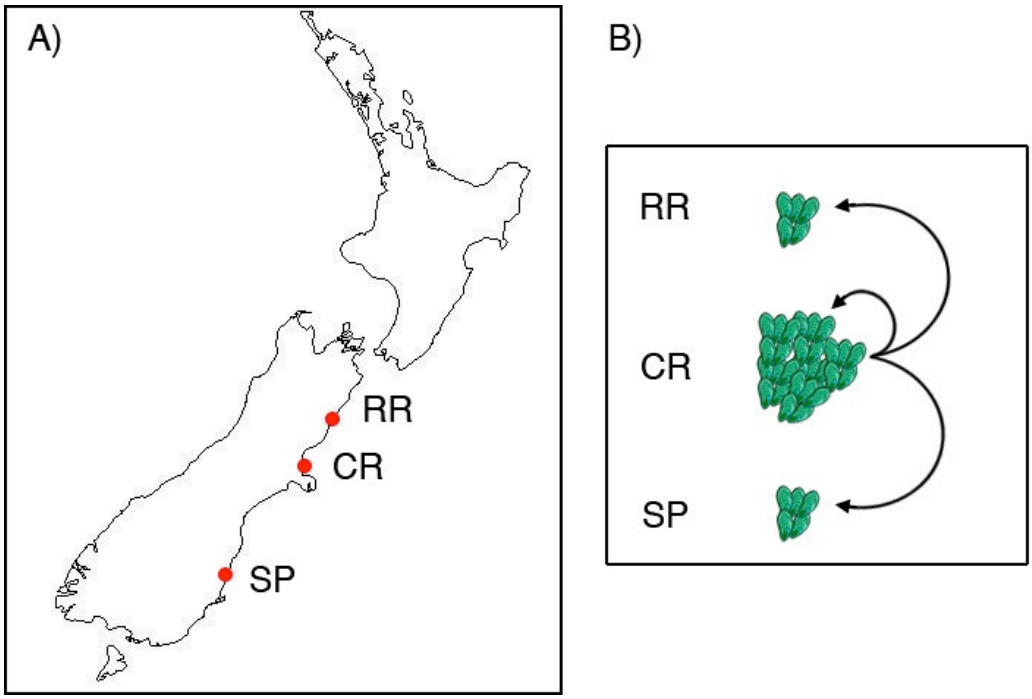


Figure 4.1 Field sites for the transplant experiment. A) Map of field sites. RR, Raramai, CR, Cave Rock, SP, Shag Point. GPS coordinates are in Table 1.1. B) Schematic of experimental design. *P. canaliculus* were transplanted from CR to RR and SP and back into CR.

4.2.2 Transplants

Approximately 325 *P. canaliculus* (40-60 mm total length) were collected from a source population at Cave Rock (**Figure 4.1A**). The mussels were transported to the University of Canterbury in an ice chest with dividers that kept them from direct contact with the ice. All mussels were then measured and tagged individually (Hallprint 8 × 4 mm tags; **Figure 4.2**). The mussels were haphazardly separated into groups of 25 before being wrapped in squares of shade cloth and tied with a zip tie to keep them organised in groups. The mussels were kept overnight in an aquarium with flow-through seawater before being transplanted to field sites. The following day, three research teams brought 100 mussels to each of the three sites. As a transplant control, mussels were also brought back to the source site, Cave Rock.

Figure 4.2 Photo of a tagged *P. canaliculus* used in the experiment.

To avoid effects of transport stress differentially affecting mussels at the three sites, all mussels were removed from the aquaria and placed into ice chests at the same time on the day of transplant. The mussels were then transplanted to their respective sites on the same low tide; therefore all mussels were in 'transport' conditions for approximately the same amount of time.

Mussels were transplanted into similar tidal elevations (**Table 4.1**) at the three sites following established methods (Menge, 1992; Menge *et al.*, 1999; Menge *et al.*, 2007; Menge *et al.*, 2008). Three plots of 25 mussels (75 mussels in total) were placed ventral side down onto bare rock that had been cleared of biota. The plots were covered with vexar mesh held to the rock with plastic washers and lag screws. Plots were left for one month to allow mussels to re-attach and then mesh was removed. The plots were monitored monthly from December to April and gill tissue samples were collected (described below); any dead shells were removed and measured. In April 2010, mussels in all the plots were collected, their total lengths measured and tissue samples collected.

Table 4.1 Tidal elevation of experimental plots at the three sites, metres above mean sea level (mean ± SE).

Site	Tidal elevation (m)
Raramai	1.02 ± 0.02
Cave Rock	1.10 ± 0.02
Shag Point	1.19 ± 0.01

Approximately 15 mussels at Cave Rock, the control site, were measured and tagged *in situ* to assess growth and gene expression of undisturbed mussels

(Chapman, 1986). Unfortunately, none of these mussels could be located at the end of the experiment. When tagging mussels *in situ*, shellfish tags were glued to the shell and it is possible that the glue may have not had time to set before the subsequent high tide submerged the mussels. Growth comparisons between non-transplanted, undisturbed control mussels and transplanted individuals could not be made due to the lack of *in situ* tagged mussels at the end of the experiment.

Gill tissue samples were collected monthly from one mussel per plot (n = 3 mussels for each treatment [site and time]) and from non-transplanted wild type control mussels at Cave Rock. In the field, mussels were opened by severing the adductor muscles and the gill was removed. Each gill was placed into a 1.5 ml tube and dropped into liquid nitrogen to flash-freeze the tissue. Samples were brought back to the laboratory and stored at −80˚C until processed.

4.2.3 Molecular methods

Mussel tissue was prepared for RT-qPCR by RNA extraction and cDNA synthesis through various methods described in detail in Chapter Two. Relative expression of *hsp70* in *P. canaliculus* was tested in animals that had been transplanted between the three sites. HSP70 was the only gene used because amplification of other target genes was unsuccessful due limited sequence conservation between *Perna* and *Mytilus* (explained in **Section 2.2.7**). RT-qPCR from three pooled biological replicates was done in triplicate on a Rotor-Gene Q (Qiagen) machine. Two stably expressing reference genes (*18S* and *actin)* were used in this study (primer sequences available in **Table 2.1**). The RT-qPCR amplification of *hsp70* was normalised to the two reference genes.

4.2.4 Temperature recording

A Tidbit™ temperature logger (Onset Computer Corp., Pocahassett, MA, USA) was deployed in the low intertidal zone among the experimental plots at each site (Helmuth and Hofmann, 2001; Menge *et al.*, 2003; Petes *et al.*, 2008). The surface temperature was recorded every 10 minutes for the duration of the experiment. Temperature data were coupled with tide tables to separate air and water temperatures. The daily emersion time at each site was calculated using the decoupled air and water temperature readings.

A temperature stress index (TSI) was calculated to examine the effects of temperature and emersion time together. The TSI is the sum of temperatures

during air exposure. A higher TSI would result from either longer air exposure (more temperature readings to sum) or hotter temperatures during air exposure (greater temperature values to sum).

4.2.5 Data analysis

All data analyses were done in R statistical software (Version 2.14.1; R Development Core Team, 2011). Differences in the proportion of mussel survival between sites and the effects of temperature on survival throughout the course of the experiment were tested with a generalised linear model (GLM). The GLM used a logit link function and quasi-binomial distribution to account for overdispersion. The test factors 'site' and 'time' were analysed as fixed factors and 'temperature' was a categorical variable. A likelihood ratio test (LRT) with a Chi-Squared distribution was run on the GLM results.

Growth was calculated as the difference between final and initial lengths. Data analysis on mussel growth at the different sites was done using a GLM with a Tweedie distribution. By setting the Tweedie index parameter $p = 1.2$, a Poisson-Gamma distribution was generated that is ideal for representing a continuous variable with an overabundance of zeros such as the growth measurements from this experiment (Shono, 2008). Tests of multiple means were done using Tukey's HSD.

RT-qPCR data analysis was done using a modified Pfaffl equation to account for multiple reference genes (**Equation 2.2**) (Pfaffl *et al.*, 2004; Hellemans *et al.*, 2007). Biological triplicates were pooled before RNA extraction took place and qPCR was run in technical triplicate. Site-wise differences were analysed with Monte Carlo randomisations on the mean fold change of gene expression, relative to non-transplanted wild type control samples from the source site, Cave Rock. The gene expression at each time point was set relative to samples from non-transplanted wild type control mussels at the origin site, Cave Rock, at the same time point. By comparing expression in this way, it was possible to examine expression patterns in the control transplant mussels and also compare them to the transplanted mussels at the two different sites.

4.3 Results

4.3.1 Temperature

Maximum air temperatures were variable across sites and months (**Figure 4.3**). The hottest temperature spikes were at Raramai for the duration of the experiment. Cave Rock and Shag Point showed a decline in temperature after February. Raramai consistently had the highest ranked monthly average air temperature among the three sites (**Table 4.2**) as well as the highest frequency of hot days (**Figure 4.4**). Daily temperatures reached as high as 36˚C at Raramai, 32˚C at Cave Rock, but only 28˚C at Shag Point (**Figure 4.4**). The temperature differences between sites follow a latitudinal trend, with the coolest temperatures being found at the most southern site, Shag Point.

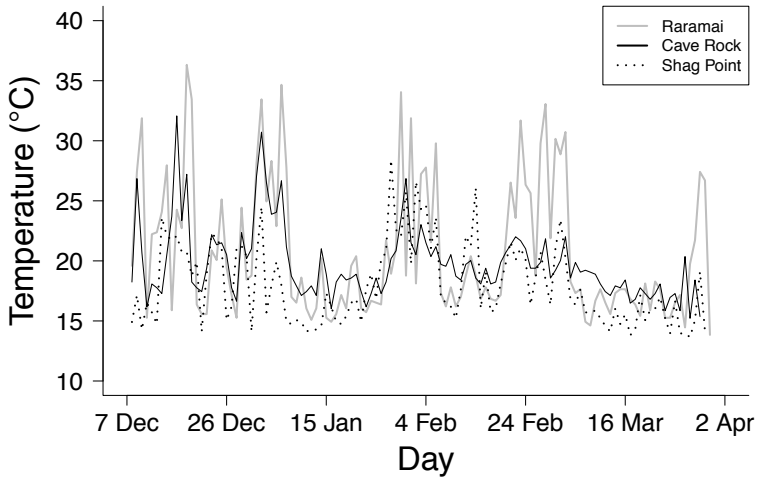


Figure 4.3 Daily maximum air temperature at each site, December 2009 to April 2010.

Table 4.2 Average monthly maximum air temperature (˚C, mean ± SE).

	Dec	Jan	Feb	Mar
Raramai	22.02 ± 1.18	20.47 ± 1.07	22.18 ± 1.06	18.69 ± 0.81
Cave Rock	20.73 ± 0.75	20.37 ± 0.69	20.15 ± 0.25	17.97 ± 0.27
Shag Point	18.77 ± 0.63	17.84 ± 0.67	19.77 ± 0.61	16.14 ± 0.4

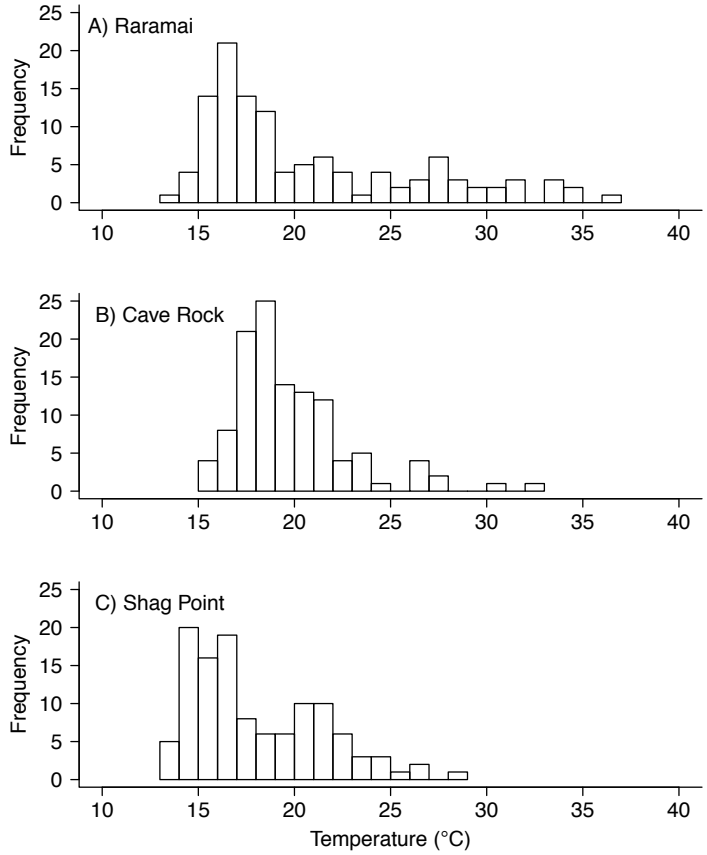


Figure 4.4 Frequency histogram of daily maximum air temperature for each site over the whole experiment. A) Raramai, B) Cave Rock, C) Shag Point.

4.3.2 Emersion time

For approximately the first two months of the experiment, December 2009 – January 2010, mussels at the three sites were submerged for about the same amount of time each day (**Figure 4.5**). As the summer progressed, Cave Rock was exposed to air for less time than the other two sites, and Raramai the most (**Figure 4.6**). With only small differences in elevation (**Table 4.1**), it is unlikely that the emersion times were driven by elevation; site topography, such as the angle of the rocky bench, or the presence/absence of boulders affecting wash splash patterns, would probably have had a greater influence on exposure than simply tidal elevation.

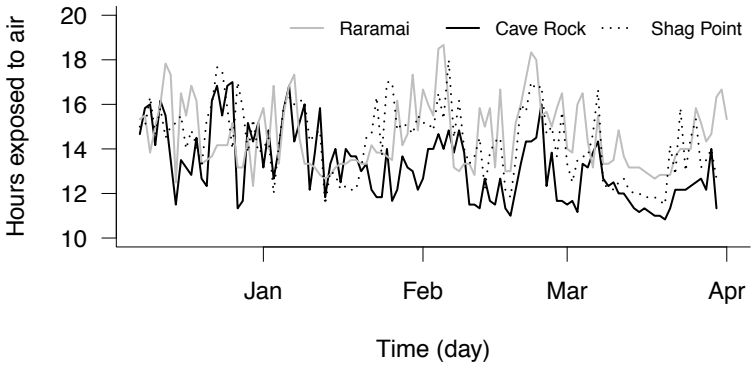


Figure 4.5 Number of hours day^{-1} that the transplanted *P. canaliculus* were exposed to air at the three different sites.

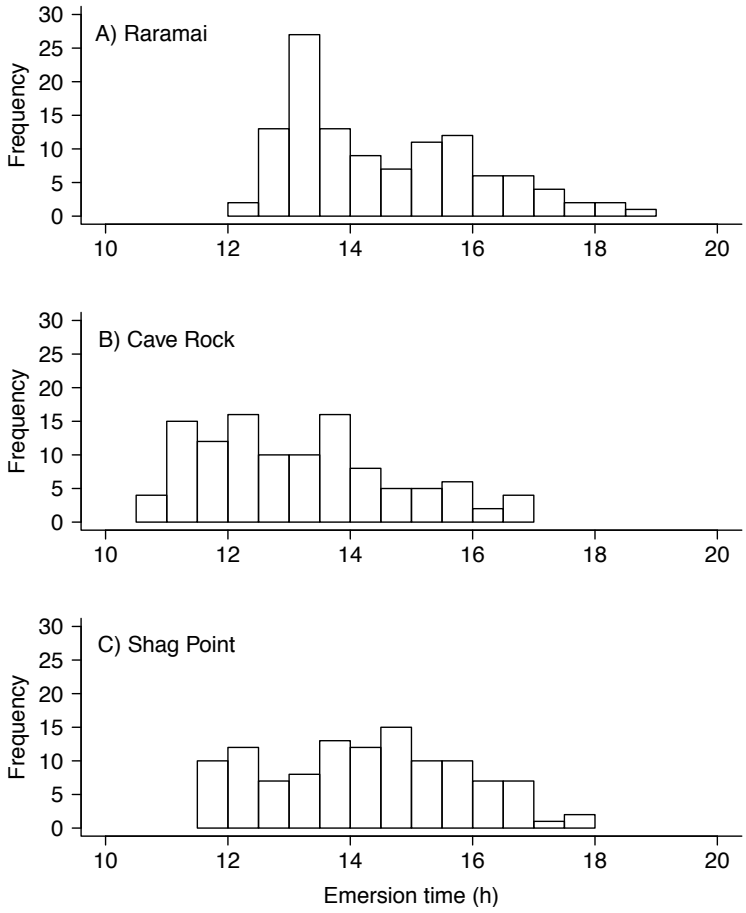


Figure 4.6 Frequency histogram of daily emersion time (h) for each site over the whole experiment. A) Raramai, B) Cave Rock, C) Shag Point.

Based on temperature profiles, it would be expected that the strongest stress response would be at Raramai and the weakest at Shag Point, especially if days with long air exposure happened to coincide with days of high air

temperature. A correlation was done between emersion time and maximum daily air temperature to test if these days coincided (**Figure 4.7**). At Raramai, there was a slight correlation, but this correlation is weakened by the large amount of variation. Additionally, when emersion time is low the propensity to heat up is low as shown by the lower variability in points at low emersion times. The window of emersion time (hours day^{-1}) used in the correlation was for daytime exposure, between 06:00 and 21:00 hrs. This time window encompasses the time when thermal conditions are higher and thus potentially hazardous. Several different time windows were trialed by varying the morning and evening times but there were no appreciable differences in the results between the different time windows, thus the time window that encompassed all the daylight hours in the height of summer was chosen.

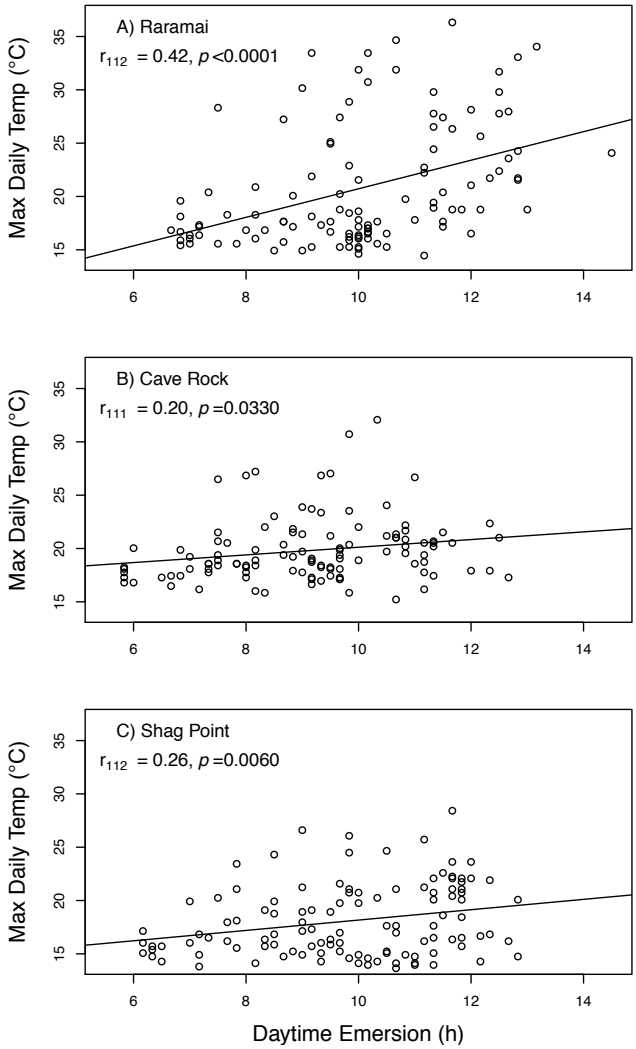


Figure 4.7 Correlation of emersion time and maximum daily temperature. Each point represents the maximum temperature for one day of the experiment and for how long that day the mussels were exposed to air (Dec 2009-Apr 2010; 112 days). Each panel represents a different site, A) Raramai, B) Cave Rock, C) Shag Point.

4.3.3 Temperature stress index (TSI)

The early summer (December, January) TSI was similar at Cave Rock and Raramai, but in the hottest months (February, March) TSI at Raramai was the highest (**Table 4.3**). Shag Point TSI was the lowest in all months. Overall, there was a decrease in the TSI from north to south.

Table 4.3 Monthly and overall temperature stress index (TSI) for each site.

Site	Dec	Jan	Feb	Mar	Overall
Raramai	24.4	29.5	31.8	27.8	243
Cave Rock	25.1	30.5	28.1	24.9	227
Shag Point	22.2	26.6	27.6	23.3	209

4.3.4 Survival

Mussels at Raramai had lower average survival than the other two sites but at all sites there was large within-site variation and so no site differences were significant (LRT, $p = 0.42$; **Figure 4.8**; **Table 4.4**). TSI was a significant factor for survival (LRT, $p = 0.002$) but the only interaction that was significant in a Tukey's HSD was not biologically meaningful (Raramai survival in March relative to Cave Rock survival in January). Temperature and emersion time were not significant factors for survival in the GLM (**Table 4.4**). A correlation between survival and temperature, emersion and the TSI was run on the data. Temperature and emersion time remained non-significant and TSI was negatively correlated with survival (**Table 4.5**).

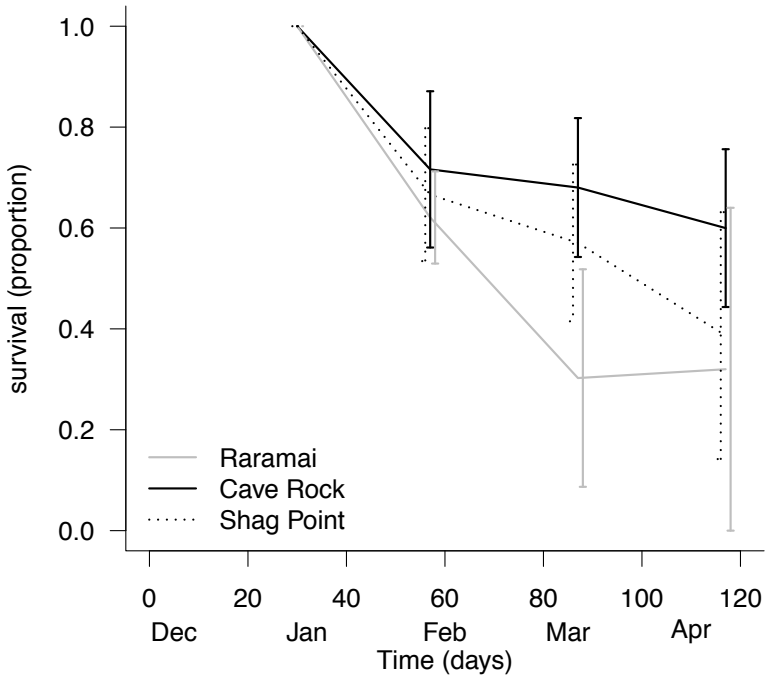


Figure 4.8 The proportion of mussel survival (mean ± SE) over the course of the experiment at the three sites. The experiment was installed in December, but the first measurements were collected in January in order to limit a bias in the survival curves resulting from death due to transplantation.

Table 4.4 Quasibinomial GLM analysis of deviance table for mussel survival testing the effects of site, time, temperature, emersion and TSI (GLM Pseudo $R^2 = 0.56$). Test factors in bold were significant ($p < 0.05$).

	LR Chisq	**df**	**Pr(>Chisq)**
Site	1.70	2	0.4280
Time	**8.84**	**1**	**0.0029**
Temperature	0.35	1	0.5564
Emersion	0.02	1	0.8935
TSI	**9.88**	**1**	**0.0017**
Site × time	0.54	2	0.7625

Table 4.5 Correlation analysis of survival with abiotic factors, average monthly maximum temperature, average monthly emersion time and temperature stress index (TSI), which was the sum of all temperature readings during emersion. Test factors in bold were significant ($p < 0.05$).

Correlation	r	df	p-value
Survival × temperature	0.23	35	0.18
Survival × emersion	0.16	35	0.16
Survival × TSI	**−0.38**	**35**	**0.02**

4.3.5 Growth

Mussel growth varied significantly among the three sites ($F_{2, 65} = 197.98$, $p < 0.0001$, $R^2 = 0.85$, **Figure 4.9**). Through the course of the experiment, the transplant control mussel growth rate at Cave Rock was substantially more ($1.9 \times 10^{-3} \pm 1.15 \times 10^{-4}$ mm day^{-1}) than at Raramai and Shag Point where the mussels grew very little ($2.89 \times 10^{-4} \pm 5.35 \times 10^{-5}$ mm day^{-1} and $3.57 \times 10^{-5} \pm 1.44 \times 10^{-5}$ mm day^{-1} respectively). Many mussels did not grow at all at Shag Point or Raramai. Mussels at Shag Point had the lowest range of temperatures and were emersed for longer than at Cave Rock but less than Raramai. The TSI was not significantly correlated with growth at any site ($r_1 = 0.29$, $p = 0.81$).

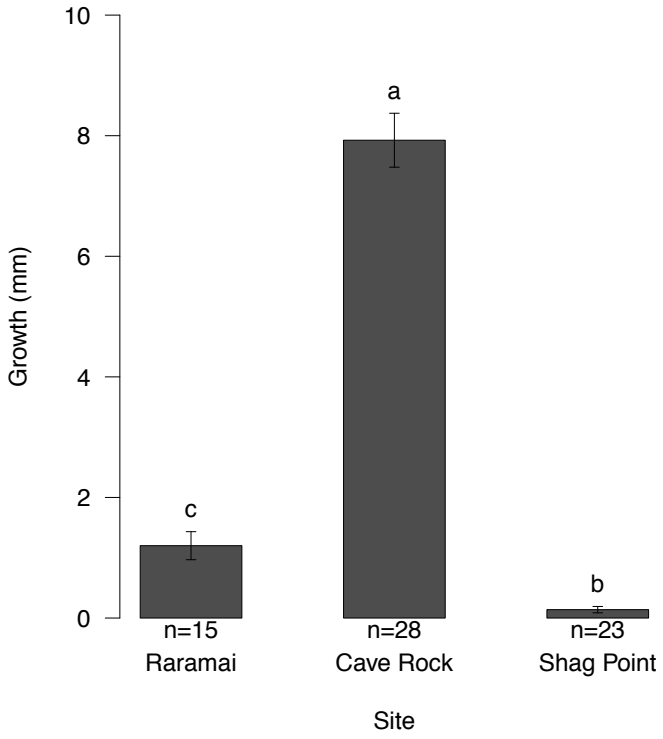


Figure 4.9 Absolute growth (mm ± SE) of *P. canaliculus* at three sites, Raramai, Cave Rock and Shag Point at the end of the summer. Different letters denote significant difference, $p < 0.0001$.

4.3.6 Gene expression

Over the course of summer, upregulation generally increased in the last two months (Figure 5.10). The greatest response was at Raramai and the least at Cave Rock. The expression of *hsp70* in *P. canaliculus* transplant-control mussels at Cave Rock was only slightly upregulated but not significantly different to non-transplanted wild type control mussels (**Figure 4.10**). Expression was significantly upregulated at Raramai relative to non-transplanted wild type control animals from Cave Rock (Monte Carlo randomisation, $p < 0.05$, **Figure 4.10**). At Shag Point there were incremental steps in *hsp70* expression level in February and April in the amount of upregulation (**Figure 4.10**) but the expression was not significantly different than the Cave Rock animals. Raramai expression increased throughout the summer to a maximum of 8.52 ± 0.22–fold change (mean ± SE) greater than non-transplanted wild type control mussels from Cave Rock at the end of the experiment in April, the highest *hsp70* upregulation of any site for the duration of the experiment.

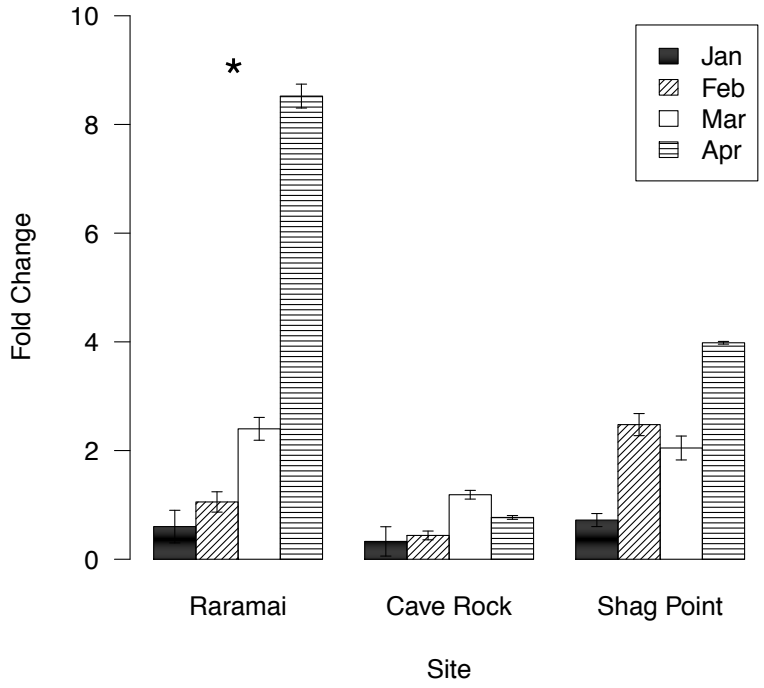


Figure 4.10 *P. canaliculus* gene expression of *hsp70* at each site relative to non-transplanted wild type control mussels from the source population of Cave Rock. Asterisk denotes that expression at Raramai was significantly different to the other sites (Monte Carlo randomisation, $p < 0.05$).

The analysis took into account any natural variability in gene expression that may have occurred in the population over the course of the summer. Cave rock non-transplanted wild type control mussels had slight downregulation of *hsp70* expression for the duration of the summer (**Table 4.6**). The amount of upregulation shown in **Figure 4.10** represents the upregulation at each of the three sites relative to the expression at the same specific time at the control site of Cave Rock. The Cave Rock samples are the transplant control samples that were removed from the rock and transplanted back to the same site. The slight upregulation shown by the samples at Cave Rock thus represents the natural expression throughout the summer.

Table 4.6 Fold change of *hsp70* expression of Cave Rock non-transplanted wild type control mussels showing that the expression in Cave Rock mussels varied very little over the course of the summer. Expression was set relative to the January samples, therefore no expression value is assigned for that month.

	January	**February**	**March**	**April**
Cave Rock (Control)	–	-0.02 ± 0.07	-0.15 ± 0.16	-0.42 ± 0.04

4.3.7 Results summary

Raramai was ranked as the most stressful site and Cave Rock as the least (**Table 4.7**). Mussels at Raramai had the highest *hsp70* gene expression, low survival and low growth, while Cave Rock mussels had low *hsp70* expression, good survival and high growth. There were longer emersion times and higher temperatures at Raramai. Shag Point was the coolest site, and was emersed for less time than Raramai but showed negligible growth. Significant differences in growth and gene expression at the transplant site, at Raramai in particular suggest that sublethal stress was an important factor between the different sites.

Table 4.7 Summary table of gene expression (*hsp70*), total growth (mm), proportion survival, average daily maximum temperature (˚C, mean ± SE), average daytime emersion (hours, mean ± SE), the temperature stress index (TSI: the sum of the temperature readings during air exposure) and the overall stress ranking, where 1 is the most and 3 is the least stressful site.

Site	*hsp70*	Growth	Survival	Temperature	Emersion	TSI	Rank
Raramai	8.52	1.20	0.32	20.75 ± 0.53	10.02 ± 0.17	161	1
Cave Rock	0.77	7.93	0.60	19.80 ± 0.28	9.12 ± 0.16	154	3
Shag Point	3.98	0.14	0.39	18.08 ± 0.32	9.82 ± 0.17	141	2

4.4 Discussion

There were variable responses of mussels among the three transplant sites. The major conclusion is that in the most stressful conditions, there is poor survival, upregulation of HSP, and generally poor growth, which are signs of environmental stress (Hofmann and Somero, 1995; Feder and Hofmann, 1999). There was clearly wide variation in most measures both within and between sites, which obscured some of the patterns across sites. It may well be the case that such within-site variability is a common feature of mussel-dominated communities. For example, it has been shown that the positions of mussels within beds can greatly affect their temperature environment and stress levels (Cole, 2010). It is significantly cooler and there is higher humidity within a mussel bed than outside (Cole, 2010). At the site level, food provision may also be compromised, which would certainly affect growth. For example, other studies have shown that food is limited at these transplant sites (Menge *et al.*, 2003; Bracken *et al.*, 2012). From the Menge *et al.* (2003) study, Box Thumb, a site near Cave Rock, had chlorophyll-a

levels of approximately 1.3 μgL^{-1}, while Shag Point and Raramai had levels of approximately 0.7 μgL^{-1} and 0.75 μgL^{-1} respectively. Chlorophyll-a is positively correlated with mussel growth (Menge *et al.*, 2003; Bracken *et al.*, 2012). Emersion time and temperature were higher at one transplant site (Raramai). Mussels at Raramai had elevated *hsp70* expression and low growth. Mussels at the other transplant site (Shag Point), which also had low food input, had almost negligible growth but *hsp70* expression was only slightly elevated. Mussels at the control-transplant site (Cave Rock) grew substantially more than the other sites and did not upregulate *hsp70* expression. Chlorophyll-a levels were not collected in this study and having direct measures of food while the experiment was running would have been beneficial. However, the studies by Menge *et al.* (2003) and Bracken *et al.* (2012) that did measure chlorophyll-a levels at the same, or nearby, sites support the stress response data that was collected here. These results suggest that the mussels at Raramai and Shag Point may have been negatively affected physiologically by suspected limited food.

A combination of multiple stressors can produce an elevated stress response. Schneider (2010) has shown that when thermal and nutritional stressors are compounded, *M. galloprovincialis* survival is highly compromised. In my study, the effects of apparent low food availability coupled with high temperatures at Raramai were reflected in the comparatively high expression of stress indicator gene, *hsp70*, in *P. canaliculus.* Shag Point, the southern site in this study, has similar oceanographic food conditions to Raramai (Menge *et al.*, 2003) but with less extreme temperatures. *Hsp70* expression at Raramai was higher than at Shag Point, this is likely due to the higher temperature and emersion times at Raramai inducing higher physiological stress. The *hsp70* protein is a molecular chaperone that interacts with other proteins that are not in their native conformation, thus preventing aggregation of damaged proteins and aiding in their repair (Feder and Hofmann, 1999). Upregulation in mussels from Raramai indicated that mussels were experiencing conditions that were compromising the integrity of their protein pool. Gene expression of mussels transplanted back to the source site, Cave Rock, were not different to undisturbed mussels from Cave Rock (**Table 4.6**).

Physiological adaptation through changes in gene expression is an important mechanism for coping with environmental stress. There was increased *hsp70* expression in this study, which was possibly at the expense of growth.

Upregulation of HSPs can be metabolically costly and at the expense of other cellular functions (Krebs and Holbrook, 2001). The extent to which an organism can mount a response to protein damages through environmental stress is known to fluctuate (Buckley *et al.*, 2001; Ioannou *et al.*, 2009; Tomanek, 2010). In some cases the protein damage is too severe, which can lead to damaged proteins being shunted into the ubiquitin pathway and slated for destruction (Feder and Hofmann, 1999). If the damage is less serious, then the upregulation of chaperone proteins can be the desired route for repair. This is due to the amount of energy required for repair of proteins tends to be less than the combined metabolic costs of destruction followed by new protein synthesis (Kassahn *et al.*, 2009). However, shunting of energy to upregulate chaperone proteins could come at the expense of other cellular functions such as growth (Feder, 1996) and reproduction (Krebs and Loeschcke, 1994).

Long term survival may be compromised at sites with high physiological stress by surpassing a tolerance threshold. There was greater upregulation of *hsp70* in mussels from Raramai as the summer progressed, indicating that the need for repairing protein damage increased through time. There were differences in growth and gene expression between sites, which were likely due to the environmental conditions present at each site. This indicates that the mussels from one population are able to respond differently to altered environmental conditions. Although there were no significant differences in survival, average survival at the three sites ranged from 35-65%. Long term survival may be compromised for mussels populations at stressful locations such as Raramai and to some extent at Shag Point due to the combination of thermal stress, emersion and potential nutritional stress (Schneider *et al.*, 2010). Predicted climate change related conditions are likely to present organisms with novel environments (Christensen *et al.*, 2007; Zippay and Helmuth, 2012) and the persistence of a population in this new set of conditions is unclear (Chown and Gaston, 2008; Zippay and Helmuth, 2012). Given the elevated stress response seen here at Raramai, long-term persistence of the population in environmental conditions of this sort would be unlikely.

Stressful factors can be either biotic or abiotic. Biotic factors such as predation can cause significant stress to mussels. For example, increased heart rate is a measure of stress (Rovero *et al.*, 1999; Stenseng *et al.*, 2005; Braby and Somero,

2006). Heart rates increase in mussels when exposed to the effluent of a predatory gastropod, the whelk *Nucella lapillus* (Rovero *et al.*, 1999). At the sites used in this study, predatory macroinvertebrates (whelks and sea stars) were rare. Fish and crab predation, while prevalent, is restricted to mussels smaller than 30 mm (Menge *et al.*, 1999; Menge *et al.*, 2002; Menge *et al.*, 2003; Rilov and Schiel, 2006b). Mussels in this study ranged from 40-60 mm total length, and so predation is unlikely to have played a role in the stress response observed.

This field experiment was designed and done during the summer of 2010-2011 while the qPCR laboratory work was completed in 2012. The original plan was to examine the expression of multiple stress response genes (detailed in Chapter Two, **section 2.2.8**) in *P. canaliculus*. However, due to difficulties with qPCR optimisation in *P. canaliculus*, only one gene of interest (GOI) was used, *hsp70*. Multiple GOIs would have been preferable for the interpretation of the stress responses of the transplanted mussels. The lack of multiple genes for interpretation of stress responses is unfortunate in this case, however the gene that was used is the quintessential stress response gene. Species-specific behavioural or biochemical changes typify many types of stress response. However, *hsp70* upregulation is a universal phenomenon (Gross, 2004). In virtually all organisms studied, except for a selection of cold-adapted Antarctic species (Hofmann *et al.*, 2000), HSP70 gene or protein expression levels are nearly ubiquitously found to be upregulated in response to stress (Feder and Hofmann, 1999).

Any transplant experiment should have several sets of controls included in the experimental design (Chapman, 1986; Honkoop *et al.*, 2003). Three sets of controls were used in this study: transplantation, gene expression and growth. The transplantation control involved collecting mussels from the source site, and transplanting them back to the source site, subject to the same type of handling as mussels transplanted to the other locations. Gene expression controls consisted of collecting non-transplanted mussels each time experimental samples were collected for gene expression analyses. The qPCR gene expression data was then calculated relative to the non-transplanted mussels (**Table 4.6**). Growth control mussels were measured *in situ* without disturbing the mussels. To my knowledge, there are no studies that have used undisturbed mussels in growth analyses as suggested by Honkoop *et al.* (2003). Measurement of mussel size *in situ* is a very difficult task, as calipers do not fit well in a mussel bed to allow accurate

measurement of shell length. When studying other molluscs, such as snails or limpets, measurements of undisturbed animals is much easier and should be included in the experimental design (Honkoop *et al.*, 2003). I was able to measure approximately 15 mussels *in situ*, but the source site growth controls were lost during the course of the experiment. The transplant and gene expression controls were successful.

A strength of this study was that all the mussels used here originated from one population. Differences seen could be attributed to the conditions in which the animals were living, rather than potential population-level responses triggered by genetic differences. With the single source population, my study illustrates the ability of adult mussels to adapt to increased levels of physiological stress following transplant to new geographic locations. This study simulates the effects of climate change as animals will experience a variety of novel environmental conditions that may be deleterious. Here it was shown that prolonged elevated stress levels in experimental mussels at Raramai will likely result in their eventual local extinction. Inability of transplanted mussel to cope with increased stress indicates their innate physiological plasticity is insufficient to cope with the possible future effects of climate change.

Chapter Five:

Temperature effects on stress response gene expression in *Mytilus galloprovincialis*

5.1 Introduction

Physiological responses of organisms to environmental stressors are often labile, allowing the organism to respond to local environmental changes (Tomanek, 2010). Understanding the limits of physiological adaptations becomes increasingly important as the climate warms. Physiological performance in response to stress can vary greatly depending on the recent thermal history of an organism (Buckley *et al.*, 2001). For instance, animals adapted to warm conditions can withstand higher temperatures without initiation of a heat shock response (Feder and Hofmann, 1999). When thermal stress does require a response, animals can produce heat shock proteins (Hsps) to stabilise proteins inside cells and to help cope with environmental stress. Long-term acclimation to stress can be achieved by changes in the structure of cellular machinery by modifications in the interactions between amino acids in proteins to enable superior function in less-than-favourable conditions (Petes *et al.*, 2008; Somero, 2011). Animals able to limit thermal sensitivity in thermally challenging habitats have an advantage because the heat shock response is a metabolically costly process that can reduce reproductive output (Feder, 1996) and an individual's competitive ability (Sorte and Hofmann, 2004; Kassahn *et al.*, 2009).

In this chapter, the characteristics of gene expression in heat shock responses were assessed in a thermal stress experiment using tanks of varied temperature on *Mytilus galloprovincialis* to evaluate the magnitude and duration of the cellular response to acute and chronic thermal stress. Sustained elevated thermal conditions allowed the analysis of the process of acclimation. The timing, duration and magnitude of stress response gene expression were studied.

Mussels were fully immersed for the duration of the experiment to ensure that any physiological responses identified would be directly attributable to thermal aquatic stress. Air exposure could confound the results with the potential effects of desiccation. Furthermore, there are numerous studies that use similar experimental designs with immersed organisms to test physiological responses of mussels (Anestis *et al.*, 2010b; Anestis *et al.*, 2010a; Jones *et al.*, 2010; Lockwood *et al.*, 2010; Logan *et al.*, 2012).

These study temperatures were set to induce thermal stress over a temperature gradient. A mussel thermal stress study by Anestis *et al.* (2007) showed that after two weeks at 30˚C and 28˚C there was 80% and 20% mortality

respectively. The temperatures in the present study were chosen with the intent to induce stress but not result in high mortality. The hot tank was set to 26˚C, but over the course of the experiment the temperature was actually 25˚C. Additionally, the present study was done during the winter months, with ambient thermal conditions of approximately 11˚C. A thermal shock that was too great may have lead to high mortality due to mussels having the inability to adapt to such an extreme change in temperature.

In earlier chapters, the magnitude of fold change for gene expression was shown to be often low (< 2-fold). The experiments subjected mussels to altered environmental conditions in the natural environment in those chapters. The magnitude of fold change seen in other studies varies greatly. Expression of *hsp70* in segments of gill tissue that had been heat shocked from *M. galloprovincialis*, increased 3000-fold in some samples (Dutton and Hofmann, 2009). *Hsp24* expression increased 254-fold in heat-shocked *M. galloprovincialis* (Lockwood *et al.*, 2010). In a microarray study, where *M. galloprovincialis* were collected from the natural environment, gene expression increases ranged from 2 to 16-fold (Place *et al.*, 2008). It was therefore important to measure the effects of acute and chronic thermal stress on gene expression to validate the molecular work that was being done. This was due to the varied magnitude of responses in other studies and the low magnitude of expression changes found in the experiments in Chapters Four and Five. Additionally, it provided a 'proof of principle,' to ensure that these genes in New Zealand mussels were responding to thermal stress as expected compared to results of microarray studies from the USA (Place *et al.*, 2008; Lockwood *et al.*, 2010).

Gene expression of several stress response genes can be used as a measure of the magnitude of the stress response (Feder and Hofmann, 1999). Rapid changes in the expression of shsp (*hsp24)* are expected relative to more delayed gene expression changes in *hsp70* and *hsp90* (Roberts *et al.*, 2010). However, because of the quick expected response of *hsp24* to thermal stress, it was hypothesised that upregulation of *hsp24* would not be sustained for the entire duration of the three week experiment as the animals acclimated to the water temperature. *Hsp70* and *hsp90,* however, were expected to maintain the upregulation for the full length of the experiment. Due to the thermal characteristics of the environment that these mussels experienced, it was expected

that the expression of the other genes, *E74-like factor 2* (*elf2*) and *butyrate response factor 2* (*tis11d*) would be affected for the duration of the experiment.

Hypotheses tested in Chapter Three:

- Gene expression of stress response genes is upregulated when mussels are exposed to acute thermal stress;
- Speed of induction and intensity of gene expression changes will be different for different stress response genes: *hsp24* will respond rapidly to increased temperatures; *hsp70* and *hsp90* will respond slower than *hsp24* but quicker than *tis11d* and *elf2*.

5.2 Methods

This experiment used tanks with controlled thermal conditions. To examine the effects of acute and chronic thermal stress on gene expression, analyses were done using reverse transcription quantitative PCR (RT-qPCR) on five stress response genes, *hsp24, hsp70, hsp90, elf2* and *tis11d*.

5.2.1 Animal collection and tank set up

Approximately 225 *M. galloprovincialis* were collected from Cave Rock in Christchurch, New Zealand (**Figure 1.6**) and transported in iced coolers to the Edward Percival Field Station in Kaikoura. Mussel sizes ranged from 40-70 mm long. On arrival, the mussels were placed into open plastic mesh baskets that allowed good water flow into one 350 L tank filled with unfiltered seawater. Mussels were fully immersed for the duration of the experiment.

The mussels were allowed to acclimate at ambient water temperature (~11˚C) for four days. This acclimation period was chosen as it was intermediate between other mussel mesocosm studies; 24 h (Resgalla *et al.*, 2007), 48 h (Anestis *et al.*, 2010b), one week (Anestis *et al.*, 2010a), two weeks (Anestis *et al.*, 2007; Anestis *et al.*, 2008) and four weeks (Lockwood *et al.*, 2010; Connor and Gracey, 2011). To begin the experiment, on the fifth day, two sets of 75 mussels were collected randomly from the ambient tank. Each set of 75 mussels was split into three groups (25 mussels) and transferred to an experimental tank (350 L). Mussels remaining in the ambient tank (75), used for acclimation, were left undisturbed. Each group of 25 mussels was placed into a basket; thus, the three experimental

tanks each had three baskets of 25 mussels suspended in the unfiltered seawater (for a total of 75 mussels per tank).

Water pumps were used to circulate seawater vigorously around the tanks. Air stones aerated the seawater and heaters were attached to the side of each tank to control the temperature. The heaters also had a small pump and the temperature could be set with a thermostat.

Every day, 60 L of seawater from each experimental tank was replaced with the same volume of pre-warmed, unfiltered seawater. The temperature of the ambient tank was unaltered (~11˚C). In the second experimental tank, the temperature was raised to 21˚C (mid) and in the third experimental tank it was raised to 25˚C (hot). Temperatures were raised by approximately 1.8˚C h^{-1} once the mussels had been placed in the tanks. The 21˚C and 25˚C tanks were located in temperature-controlled rooms that were regulated at 21˚C and 25˚C respectively.

Mussels were fed a culture of live unicellular algae *Tetraselmis chuii* every four days. Algae were cultured at 18˚C in 20 L carboys using F/2 media and growth lights.

A Tidbit™ temperature logger (Onset Computer Corp., Pocahassett, MA, USA) was placed into each tank. The loggers recorded the temperature every five minutes throughout the course of the experiment.

Mussels remained in the temperature treatments for three weeks and tissue samples were collected at different time points by sacrificing three animals per basket (nine animals per tank) one hour after the 25˚C tank reached its target temperature. Tissue samples were again collected at 24 h, one week and three weeks after this time. The mussels were opened by severing the adductor muscles. The gills were removed and placed into 0.6 ml tubes and put into liquid nitrogen for flash freezing. Samples were taken back to the University of Canterbury and stored at −80˚C until processed.

Mussels were checked for mortality every four days. Animals that failed to close their valves in response to external stimuli were considered dead.

5.2.2 Molecular methods

For each sample, 10 mg of tissue from each of three mussels was pooled into a 30 mg sample. There were three replicates of pooled samples for each time and temperature treatment. A comprehensive description of the methods used to prepare tissue for RT-qPCR is given in Chapter Two of this thesis. Two stably

expressing reference genes (*18S* and *actin)* and five target genes were used in this study (*hsp24, hsp70, hsp90, elf2, tis11d;* primer sequences available in **Table 2.1**). The RT-qPCR amplification of the target genes was normalised to the two reference genes.

5.2.3 Data analysis

The physiological effects of acute thermal stress on *M. galloprovincialis* were assessed by examining the expression profiles of five stress response genes. All changes in expression for treatments were considered relative to the expression of the target gene in the ambient tank after one hour of exposure (i.e., the control standard was ambient at one hour). The ambient tank at one hour was unchanged from the ambient tank for the previous five days, which made it an acceptable control sample.

RT-qPCR was run in technical triplicate for the three biological replicates of pooled tissue (nine animals in total per treatment). Data analysis was done in R (version 2.14.1; R Development Core Team, 2011) using a modified Pfaffl equation (**Equation 2.2)** (Pfaffl, 2001; Hellemans *et al.*, 2007) to accommodate multiple reference genes. An ANOVA was used to compare temperature and time differences in gene expression. Multiple comparisons were made using Tukey's HSD to determine any significant differences in gene expression between temperature and time treatments, while controlling for Type I error caused by multiple tests.

Relative quantification of gene expression with qPCR is suitable only for the comparison of results between different samples of one gene of interest (Pfaffl, 2004; Bookout *et al.*, 2006; Rieu and Powers, 2009). To compare results between different genes, the concentration of the transcript levels in the standards used to determine the efficiency of the primer pair must be known *a priori* (Bookout *et al.*, 2006). Furthermore, it must be assumed that the efficiency of cDNA transcription is equal for each gene in each individual, although this is often not true due to limitations inherent in reverse transcription (Pfaffl, 2004; Bookout *et al.*, 2006). The qPCR assays in this thesis were sufficiently complex, in terms of sample number and treatment conditions, to prevent the acceptance of this assumption. As a result, the expression of different target genes cannot be directly compared between (among) each other.

5.3 Results

5.3.1 Observations

Mussels were originally placed in the ambient tank for the five-day acclimation period. They attached byssal threads to the baskets in which they were contained and to each other. Before the start of the temperature treatments all the mussels were gently detached. They were placed into the new tanks for the temperature treatments of 11˚C (ambient), 22˚C and 25˚C. The experiment lasted for three weeks and during this time no mussels attached anymore byssal threds. Additionally, there was zero mortality during the course of the experiment.

5.3.2 Temperature

Over the three week experiment, the average temperature (±SE) in the ambient tank was 11.82 ± 0.98˚C while the mid and hot tanks averaged 21.83 ± 1.27˚C and 25.31 ± 1.38˚C respectively (**Figure 5.1A**). **Figure 5.1B** shows the temperature in the three tanks in the first 12 h of the experiment as it was increased to the treatment temperature. The temperature in the ambient tank remained relatively stable.

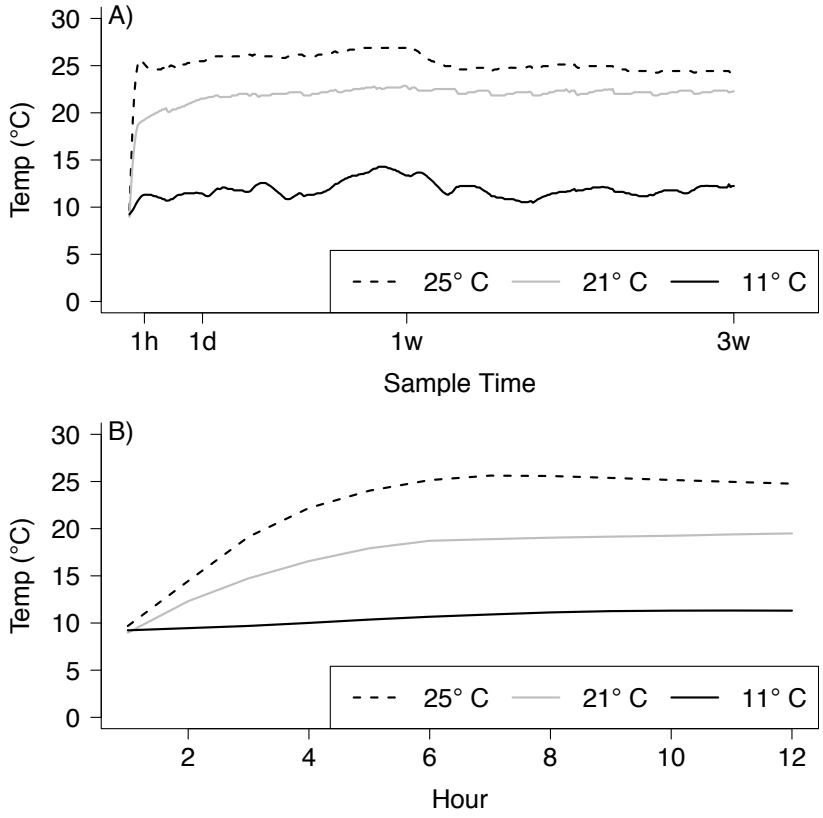


Figure 5.1 Temperature readings for the three tanks. Thermal profiles of the three tanks for the duration of the experiment. B) Heating profiles showing the heating rate in the temperature treatment tanks (21˚C and 26˚C) and the ambient tank (11˚C) for the first 12 hours of the experiment.

5.3.3 Gene expression

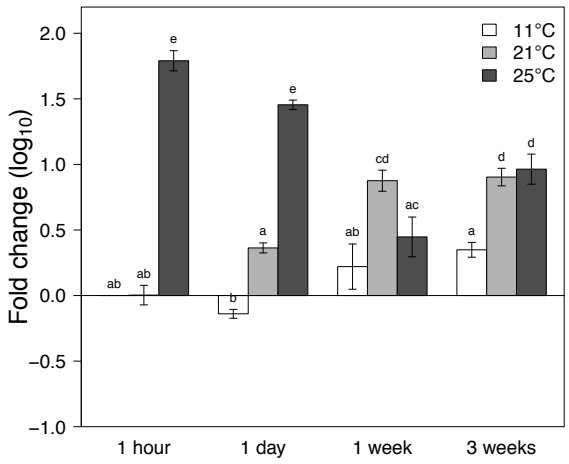
Hsp24

The expression of *hsp24* was not consistent between temperature treatments through time ($F_{6, 24}$ = 34.73, $p < 0.0001$; **Table 5.1**). Throughout the experiment, expression in the controls (ambient temperature tank of 11˚C) did not change significantly (**Figure 5.2A**). Within one hour of thermal exposure, there was substantial upregulation of *hsp24* expression in the 25˚C treatment ($10^{1.80} \pm 10^{0.08}$ relative fold change), but no significant change in expression at the 21˚C treatment ($p > 0.05$). One day after initial heating, there was still significant upregulation in the 25˚C treatment ($10^{1.46} \pm 10^{0.04}$; $p < 0.05$), while upregulation in the 21˚C treatment ($10^{0.37} \pm 10^{0.04}$) was not significantly different to the control ($p > 0.05$). After one week, the expression of *hsp24* in the 25˚C treatment was still upregulated ($10^{0.49} \pm 10^{0.15}$) but it was no longer significantly different to the control. Expression in the 21˚C treatment was significantly upregulated ($10^{0.89} \pm 10^{0.08}$) after one week. After three weeks, both temperature treatments (21˚C and 25˚C) were significantly upregulated to about the same level (21˚C: $10^{0.91} \pm 10^{0.07}$; 25˚C: $10^{0.99} \pm 10^{0.11}$ fold).

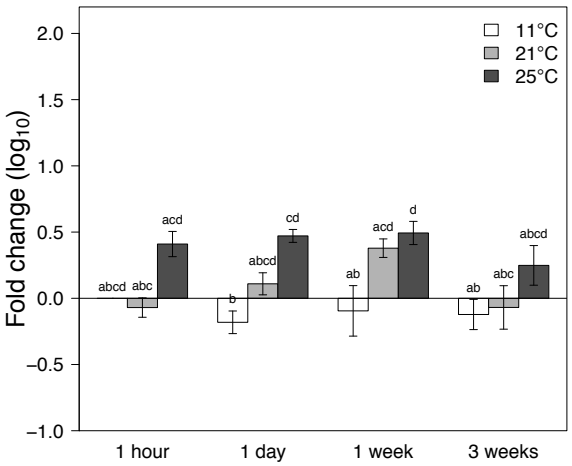
Table 5.1 Two-way ANOVA testing the effects of temperature and time on *hsp24* gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	df	MS	F	*p*-value
Temperature	**6.78**	**2**	**3.39**	**142.29**	**<0.0001**
Time	**0.25**	**3**	**0.09**	**3.55**	0.0294
Temperature x time	**4.96**	**6**	**0.83**	**34.73**	**<0.0001**
Residuals	0.57	24	0.02		

A) *hsp24*



B) *hsp70*



C) *hsp90*

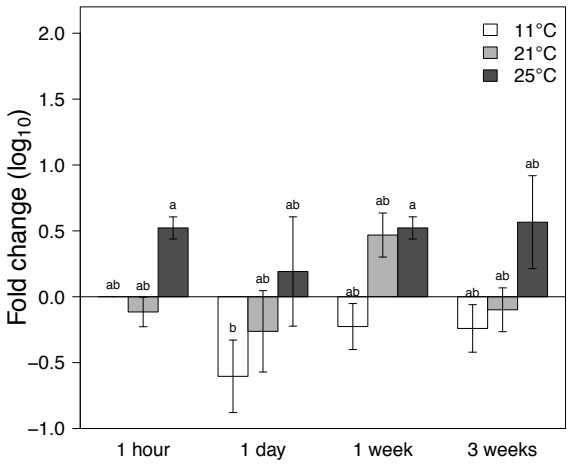


Figure 5.2 Fold change for *M. galloprovincialis* relative to the ambient control (11˚C, 1 h). A) *hsp24*, B) *hsp70*, C) *hsp90*. Samples from four time points and three temperature treatments. In each panel matching letters indicate homogeneous groups based on Tukey's HSD $p < 0.05$.

Hsp70

There was a strong effect of temperature on *hsp70* gene expression ($F_{2, 24}$ = 22.02, $p < 0.0001$; **Table 5.2**; **Figure 5.2B**). At each time point the lowest expression level was in the ambient temperature treatment and the highest expression in the hottest treatment however expression levels through time were not significant (e.g., at the one day sampling time, fold change at 11˚C was downregulated $10^{-0.16} \pm 10^{0.09}$, while 21˚C and 25˚C were upregulated by $10^{0.13} \pm 10^{0.08}$ and $10^{0.48} \pm 10^{0.05}$ fold respectively). At one day and one week after thermal exposure, the expression in the 25˚C mussels was significantly different to that in the ambient treatment at the same time points ($p < 0.05$).

Table 5.2 Two-way ANOVA testing the effects of temperature and time on *hsp70* gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	**df**	**MS**	**F**	***p*-value**
Temperature	**1.57**	**2**	**0.78**	**22.02**	**<0.0001**
Time	0.26	3	0.09	2.46	0.0868
Temperature x time	0.30	6	0.05	1.41	0.2500
Residuals	0.85	24	0.04		

Hsp90

Similar to *hsp70*, expression of *hsp90* had the highest gene expression in the two temperature treatments at all time points. There were significant temperature effects ($F_{2, 24}$ = 10.56, $p < 0.0001$; **Table 5.3**; **Figure 5.2C**). At one hour, there was an upregulation of $10^{0.54} \pm 10^{0.08}$ in *hsp90* expression in the 25˚C treatment relative to the ambient expression value. At all times there was the same pattern where the ambient treatment had the lowest expression and the highest expression occurred in the hot treatment. Expression at the mid temperature treatment (21˚C) was between high temperature and ambient values. The two increased temperature treatments were not significantly different to the control at any time ($p > 0.05$).

Table 5.3 Two-way ANOVA testing the effects of temperature and time on *hsp90* gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	df	MS	F	*p*-value
Temperature	**2.92**	**2**	**1.46**	**10.56**	**<0.0001**
Time	1.12	3	0.37	2.71	0.0686
Temperature x time	0.63	6	0.10	0.76	0.6088
Residuals	3.18	24	0.14		

Elf2

All treatments downregulated *elf2* expression for the duration of the experiment relative to the ambient control. There was a significant difference between time and temperature relative to the ambient control ($F_{6, 24} = 2.77$, $p = 0.03$; **Table 5.4**;**Figure 5.3**). *Elf2* gene expression fold change was significantly different from the control and most strongly downregulated at 25˚C (approximately $10^{-0.10}$ fold downregulation across time points). At one day, one week and three weeks, the ambient and 21˚C treatments expressed *elf2* at similar levels and were not significantly different from each other (e.g., at one week, 11˚C: $10^{-0.74} \pm 10^{0.07}$, 21˚C: $10^{-0.73} \pm 10^{0.08}$).

Table 5.4 Two-way ANOVA testing the effects of temperature and time on *elf*2 gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	df	MS	F	*p*-value
Temperature	**1.06**	**2**	**0.53**	**15.12**	**<0.0001**
Time	**0.80**	**3**	**0.27**	**7.61**	**0.0010**
Temperature x time	**0.58**	**6**	**0.10**	**2.77**	**0.0342**
Residuals	0.84	24	0.04		

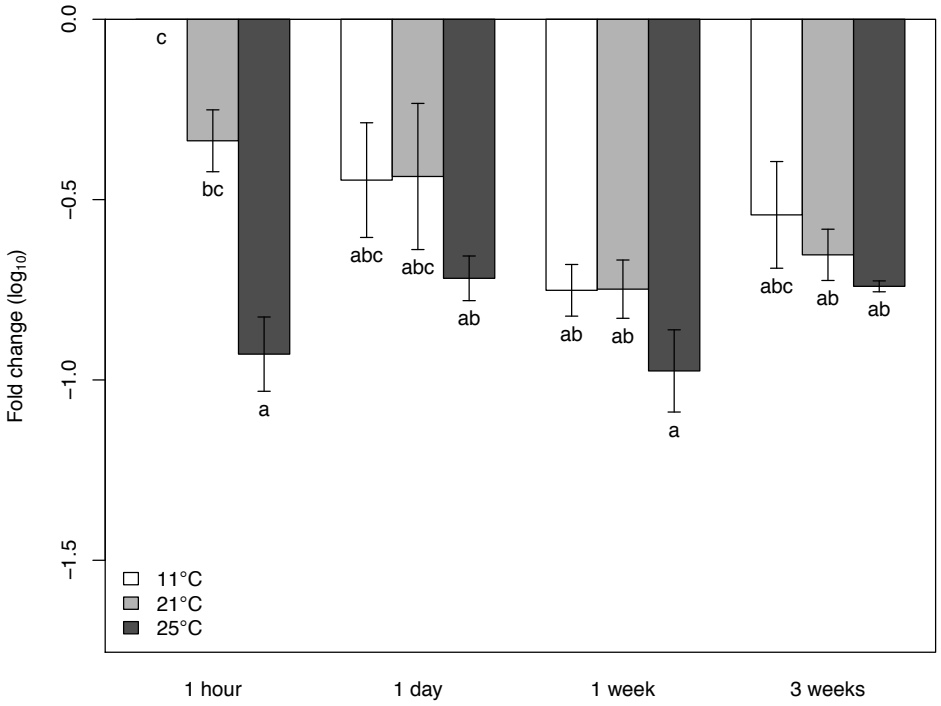


Figure 5.3 *Elf*2 fold change for *M. galloprovincialis* relative to the ambient control (11˚C, 1 h). Samples from four time points and three temperature treatments. Matching letters indicate homogenous groups based on Tukey's HSD $p < 0.05$.

Tis11d

There was limited immediate response (1 h) in the expression of *tis11d* to the heat shock experiment and expression was variable through time (**Table 5.5**; **Figure 5.4**). After one day of thermal stress, the expression of *tis11d* at 21˚C and 25˚C was upregulated similarly ($10^{0.47} \pm 10^{0.13}$ and $10^{0.43} \pm 10^{0.04}$ respectively) although this upregulation was not significant. Expression was downregulated at

one week for the ambient and 21˚C treatments ($10^{-0.25} \pm 10^{0.15}$ and $10^{-0.16} \pm 10^{0.04}$ respectively), then upregulated again at three weeks in all three temperature tanks (11˚C: $10^{0.16} \pm 10^{0.11}$, 21˚C: $10^{0.32} \pm 10^{0.13}$, 25˚C: $10^{0.45} \pm 10^{0.06}$). Overall, *tis11d* expression was variable throughout the experiment with no consistent pattern in expression change for any treatment.

Table 5.5 Two-way ANOVA testing the effects of temperature and time on *tis11d* gene expression. Test factors in bold were significant ($p < 0.05$).

	SS	df	MS	F	*p*-value
Temperature	**0.51**	**2**	**0.26**	**6.34**	**0.0062**
Time	**1.28**	**3**	**0.43**	**10.54**	**0.0001**
Temperature x time	0.40	6	0.07	1.63	0.1827
Residuals	0.97	24	0.04		

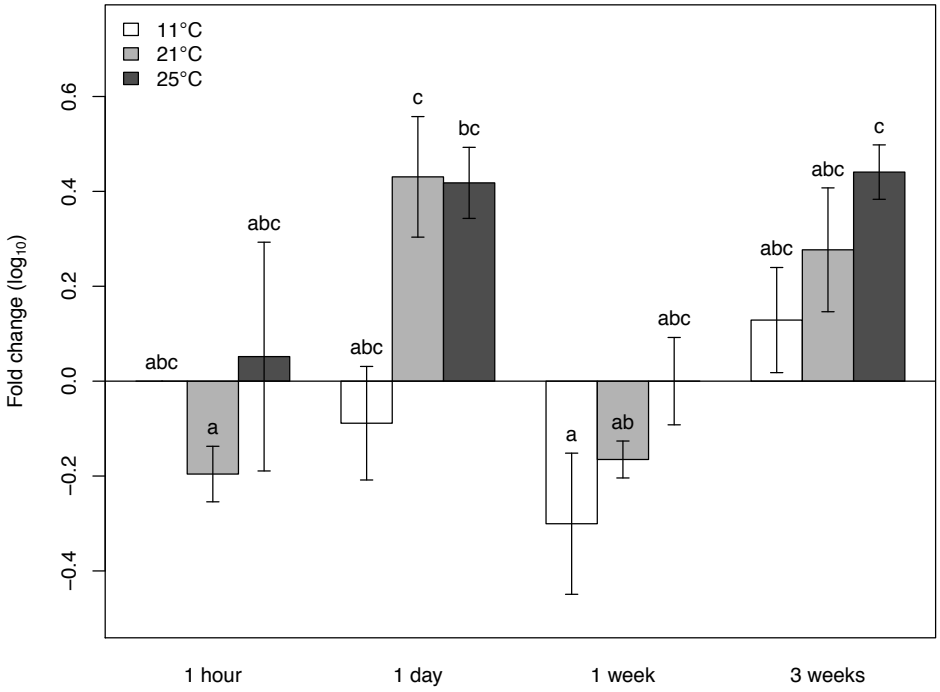


Figure 5.4 *Tis11d* fold change for *M. galloprovincialis* relative to the ambient control (11˚C, 1 h). Samples from four time points and three temperature treatments. Matching letters indicate homogenous groups based on Tukey's HSD $p < 0.05$.

5.4 Discussion

The tank experiments in this study were restricted to *M. galloprovincialis* due to the limited number of genes of interest available for *P. canaliculus*.

The three Hsps in this experiment responded similarly to acute temperature stress with upregulation at increased temperatures (**Figure 5.5**). The upregulation in all three Hsps occurred immediately upon exposure to the heat shock (at the 1 h time point) and was generally highest in the hottest temperature treatment, although not always significantly so.

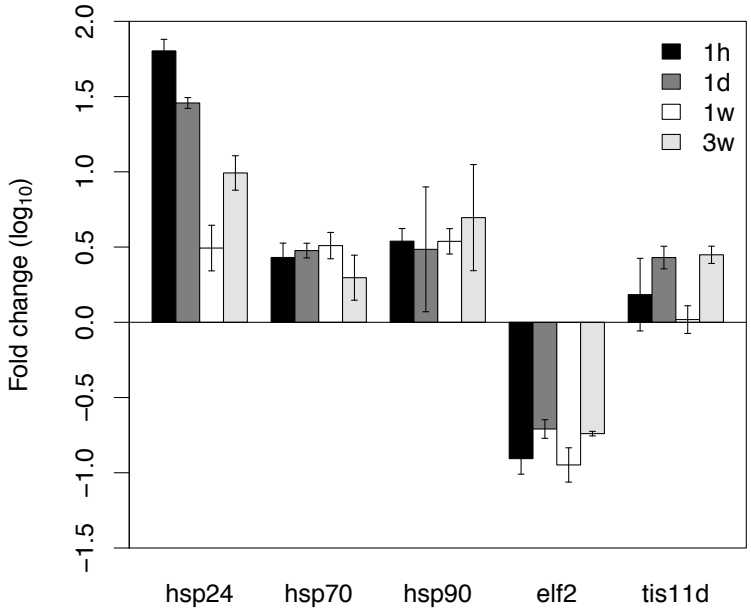


Figure 5.5 Summary of gene expression in samples from the 25˚C tank. The average fold change (±SE) of each gene relative to ambient controls (11˚C, 1 h) is included in this figure to facilitate comparisons between genes.

The small Hsp, *hsp24*, had the strongest initial upregulation that remained significant for the duration of the experiment (**Figure 5.2A**). The other two hsps (*hsp70*, and *hsp90*) maintained their upregulated level of expression at approximately similar levels for the duration of the experiment (**Figure 5.2B** and **C**). Small hsps are known to respond rapidly to environmental conditions such as thermal stress (Roberts *et al.*, 2010). The rapid upregulation is needed to stabilise damaged proteins, preventing aggregation, until other Hsps (such as *hsp70* and *hsp90*) can be produced to help repair the damaged protein (Podrabsky and Somero, 2004). Brine shrimp (*Artemia franciscana)*, whose embryos can survive for six years in completely anoxic conditions require Hsp24 chaperoning activity for their incredible survival ability (Clegg *et al.*, 1999). In the annual killifish *Austrofundulus limnaeus*, strong upregulation of small Hsps is required for survival

immediately following heat shock and before other hsps have started to upregulate their expression (Podrabsky and Somero, 2004). Small Hsps are upregulated during prolonged heat shock in the Mexican desert fish *Poeciliopsis lucida*, (Norris *et al*., 1997). Small hsps are strongly induced in response to recovery from heat stress in the mussel *Mytilus californianus* (Gracey *et al*., 2008). Sustained *hsp24* expression at all time points in the current experiment suggests there was sufficient ongoing protein damage to require the continued presence of the Hsp24 protein.

Interestingly, at 21˚C, both *hsp70* and *hsp90* were downregulated at the three-week sampling point while *hsp24* remained upregulated (**Figure 5.6**). This is in agreement with the study of desert fish whose hsp70 and hsp90 expression decreased during long term thermal stress while small hsp expression continued (Norris *et al*., 1997). The continued presence of *hsp24* suggests that proteins are still being damaged and require prevention from aggregation, but the downregulation of *hsp70* and *hsp90* at this later time suggests that physiological adaptation may be taking place in the animals through time. The mid temperature used in the experiment was meant to represent moderate thermal stress. *Hsp90* expression in the 21˚C treatment is variable, being upregulated only at the one week time point and downregulated at all other time points. *Hsp70,* however, is also most strongly upregulated at one week for the 21˚C treatment. The variable *hsp70* and *hsp90* expression suggests that the moderate stress imposed on the organisms by the 21˚C treatment is well within the animal's ability to cope physiologically.

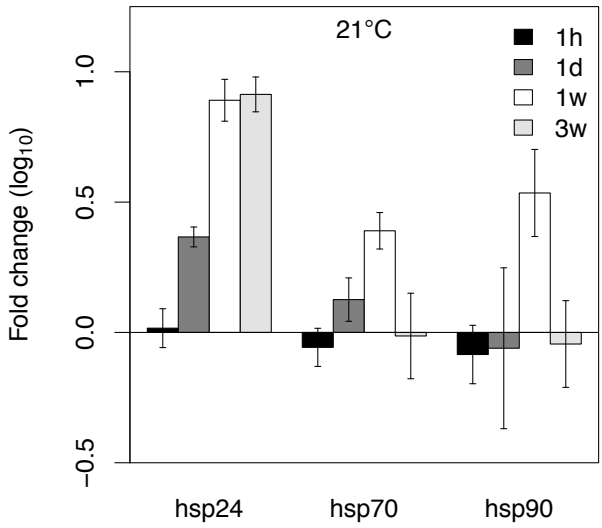


Figure 5.6 Summary of *hsp* gene expression in samples from the 21˚C tank. The average fold change (±SE) of each gene relative to ambient controls (11˚C, 1 h) is included in this figure to facilitate comparison between genes.

The specific role of Elf2 in the cell is unclear and there are relatively few studies that examine its role. There is some evidence from gene ontogeny groupings that it is involved in transcriptional regulation in mussels (Place *et al.*, 2008), while another study groups it with metabolic genes in mussels (Place *et al.*, 2012) and yet another discusses a role for *elf2* in cell proliferation in mammalian tissue culture cells (Zhang *et al.*, 2007). In stressed populations of *M. californianus*, *elf2* expression is upregulated relative to expression in mussels from less stressful locations (Place *et al.*, 2008). In the present study with *M. galloprovincialis*, *elf2* expression was downregulated and the extent of downregulation correlated with the extent of stress; the highest temperature treatment had the strongest downregulation (**Figure 5.3**). Differences in the experimental systems or upregulation of *elf2* in the control samples may be two possible explanations of the opposite expression patterns seen in the present study and the Place *et al.* (2008) study.

Differences in the experimental systems entailed *M. galloprovincialis* samples in Place *et al.* (2008) being collected during low tide, while my study used submerged mussels. There are many physiological differences in mussels exposed to air versus water. When exposed to air, heart rate decreases, body temperature increases, there is a loss of water and anaerobic metabolism is used (Bayne *et al.*, 1976). Many genes exhibit patterns of expression that are linked with tidal cycles

or air exposure and the expression patterns change with thermal stress (Connor and Gracey, 2011). Connor and Gracey (2011) showed that the expression of 24% of mussel genes in their microarray changed significantly in the 18 h that followed a simulated low-tide warming episode. To my knowledge, there is no evidence that it is physiologically stressful for intertidal organisms to be transferred to a submerged habitat.

Upregulated expression of *elf*2 in the control samples (**Figure 5.7**) could result from control mussels requiring higher *elf*2 expression. *Elf*2 is involved in transcriptional regulation of a large variety of cellular processes (Zhang *et al.*, 2007). The need to regulate a process such as growth, which is likely to be high in non-stressed mussels, may require high levels of *elf*2, while stress generally inhibits growth and metabolism and would result in a repression of those processes. Lower *elf*2 levels could repress the process since the activator is at a lower level. Indeed, cancer cells show high levels of *elf*2 expression (Zhang *et al.*, 2007), and the nature of cancer in general is that it undergoes a high rate of mitosis. In order to truly elucidate the meaning behind the response of this gene, acute thermal stress experiments should be carried out with *M. californianus* and *M. galloprovincialis* in air and water.

Upregulated *elf*2 expression could also possibly be due to the control sample being in a state of 'stress' prior to the start of the experiment. To begin the experiment, a third of the mussels in the acclimatory tank were removed to other tanks. As a result, the density in the control tank was altered. The water quality in the control tank could have been poorer during the acclimatory period due to higher mussel densities in that tank. However, the tank was quite large (350 L), and the total number of mussels was not that high (225), which gave approximately 1.5 L mussel^{-1}. This is a rather large volume per individual and therefore problems of mussel density were unlikely.

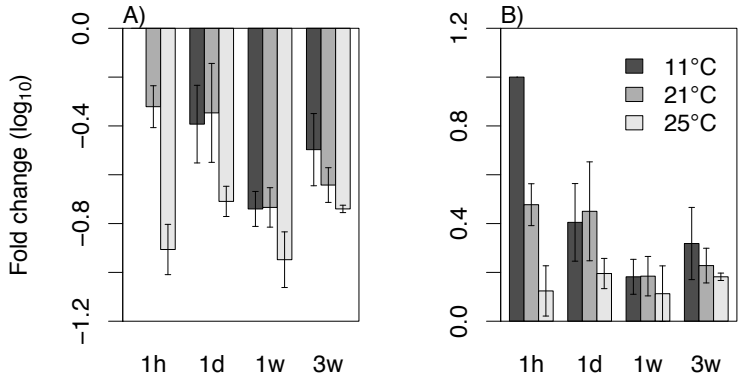


Figure 5.7 Comparing two ways of conceptualising *elf2* expression. A) Original way, relative to the control samples. B) Inverted expression, with the control being upregulated and the others being less highly expressed. The relative expression between different treatments is important, rather than the specific magnitude of expression.

Tis11d, a gene involved in regulating the cellular response to growth factors (Hudson *et al.*, 2004; Baou *et al.*, 2009), did not respond immediately to thermal stress in the tank (**Figure 5.4**). There is a variable response through time indicating that this gene may work in concert with other as yet undetermined factors to mitigate the cellular response to stress.

The sustained changes in gene expression resulting from the two temperature treatments in this study suggest that the mussels were unable to acclimate fully to the altered conditions. Changes in temperature may have been too drastic from the ambient temperature (11˚C) the mussels had been experiencing for the several months prior to the experiment, suggesting an acclimatory period of longer than three weeks would have been necessary. Also, changes from living intertidally on the rocky shore to constant immersion in this experiment may have affected the acclimation. However, it would be expected that a transition from intertidal to subtidal life would be more easily adapted to since a fully-immersed life buffers the organism from the abiotic influences of the intertidal zone. Immersion also increases feeding opportunities. The mussels in this experiment were kept in tanks with unfiltered seawater and fed every four days, allowing plenty of feeding opportunity. To be certain that mussels were coping well in the experimental conditions, the wet weight before and after the three-week treatments should have been measured. Growth was not deemed to be an important factor to measure due to the short duration of the experiment. It is unlikely that there would have been any measurable growth in such a short time

compared to the uncertainty of measurement. Therefore, in the case of this experiment, the typical stressors experienced by *M. galloprovincialis* in the field were not present and the only stress factor was temperature change. It was curious that after the acclimation period, the mussels did not reattach byssal threads in any of the tanks.

Three of five genes were upregulated in response to acute and chronic thermal stress, which partially supported the first hypothesis. One gene was downregulated, and one gene seemed to respond in no recognisable pattern. In general, the magnitude of expression change in this experiment was not high, which is not uncommon among mussel gene expression studies (Place *et al.*, 2008; Núñez-Acuña *et al.*, 2012). Therefore, larger sample sizes may have helped to decrease the error bars among the samples enabling the subtleties of expression to show through more clearly. In conclusion, temperature-induced expression of some of the genes in this study was rapid (within one hour) and sustained (for three weeks)

Chapter Six:

General Conclusions

6.1 Introduction

The aim of this study was to understand physiological responses to environmental stress used by ecologically important mussels coping with variable conditions. The biogeographic distribution of organisms is determined to a great degree by physiological characteristics that enable a population to adapt and persist in a specific location. Using field experiments, I set out to induce stress in mussels by translocating *P. canaliculus* and *M. galloprovincialis* between intertidal zones at four sites around the South Island of New Zealand. Additionally, *P. canaliculus* were transplanted from one source population to sites with different environmental conditions. Finally, a mesocosm study was done to test the effect of temperature on the expression of five target genes in *M. galloprovincialis*. My results clearly indicated that site-specific characteristics, particularly relating to high temperatures, sedimentation and possibly limited food play a major role in the intensity of the stress response and that acclimation is limited under stressful conditions, at least over a period of months. These results also call into question the factors driving mussel zonation in the rocky intertidal zone of New Zealand because *P. canaliculus* seemed to be better adapted to stress than *M. galloprovincialis*. The molecular techniques used in this project demonstrate how expression of stress-response genes can be used as a marker for physiological stress. This concluding chapter reviews the findings from the research with regard to the overarching themes of the research, and places them in context within the international literature.

6.2 *In situ* measurement of stress

Studies of organismal responses to stress have often been done in mesocosms with highly controlled conditions that allow for relatively straightforward interpretation of results. The variable of interest is individually manipulated and as many other variables as possible are held constant with a high degree of control. Field experiments attempt to manipulate only the variable of interest while permitting the uncontrolled natural variation of other variables. The control of the mesocosm enhances the precision of the experiment but there is a loss of realism, whereby variables that affect the organism may not be present (Connell,

1974). Extrapolation of mesocosm results to generalisations about the natural environment can be problematic (Petersen *et al.*, 2003). Therefore, field studies in conjunction with mesocosm experiments are beneficial to test the validity of results and the associated extrapolations in the natural environment.

Highly variable and complex field conditions can lead to difficulties in the interpretation of data from field studies. The results of field experiments presented in this thesis had primarily low fold changes in gene expression (e.g., **Figure 3.14-3.20, 4.10**). The magnitude of stress response was not as high as anticipated. Evidence from gene expression studies in more controlled conditions or less elaborate field experiments suggested that there could be large changes in expression of the selected genes. In a study overseas, for example, gill tissue from *M. galloprovincialis* collected at different sites in California and heat stressed in a water bath had up to 3000 fold increase in *hsp70* expression (Dutton and Hofmann, 2009). Additionally, *hsp24* expression in *M. galloprovincialis*, also from California was upregulated 254-fold following heat shock treatment in a laboratory setting (Lockwood *et al.*, 2010) It is possible that the relatively low gene expression changes in my field studies were the result of the overall severity of stressors being buffered by conditions in mussel communities in the natural environment (Tsuchiya and Nishihira, 1986; Cole, 2010).

6.3 Interindividual variation

Some of the differences and subtleties of gene expression may be a reflection of individual variation of mussels within and between populations. Phylogeographic studies of limpets (Goldstien *et al.*, 2006), sea stars (Waters and Roy, 2004) and mussels (Apte and Gardner, 2002; Star *et al.*, 2003; Wood *et al.*, 2007; Gardner and Westfall, 2012) have shown that there are genetic breaks in populations, generally around Cook Strait, between the North and South Islands of New Zealand but comparatively little variation within the South Island for mussels. In the studies above, for *M. galloprovincialis* and *P. canaliculus*, emphasis was primarily on the between-island genetic break and the genetic population structure around the North Island. *P. canaliculus* from Timaru were genetically unique as they had a high frequency of a North Island specific haplotype despite the presence of the southern haplotype (Apte and Gardner, 2002). Indeed, the stress response in mussels from Timaru was greater than the other sites in the

experiments from **Chapter Three**. The sequence of *hsp70* in samples of *P. canaliculus* used in these experiments was not different between sites, but the possibility of population-level variation in promoter or enhancer regions and transcription factor regulation is unknown. The possible differences in genetic structure of mussels from different sites in the experiments here may have attributed to the varying responses seen at these sites.

There are numerous studies that have found the magnitude of *hsp* expression to be different among multiple populations of the same species (reviewed in Favatier *et al.*, 1997; Otsuka *et al.*, 1997; Fangue *et al.*, 2006). For example, in the common killifish, *Fundulus heteroclitus*, genetically distinct populations from different geographic regions along the east coast of North America exhibit a significant difference in *hsp70* upregulation in response to heat stress (Fangue *et al.*, 2006). The sequence of *hsp70* in these fish is identical but the southern populations have significantly higher expression levels than the northern fish. Also, in different strains of *Drosophila melanogaster*, six of the seven *hsps* present are differentially expressed in response to heat shock (Otsuka *et al.*, 1997).

Genetic variation present between populations at different sites can lead to different responses to stress. The different sites used to study stress in **Chapter Three** may have been complicated by the presence of genetic differences between populations. However in **Chapter Four**, mussels were transplanted from one source site to the other locations. The physiological responses could not have been due to genetic differences since they were from the same original population.

Inter-individual variation in gene expression is a major concern when undertaking stress response studies. For example, in one population of *F. heteroclitus*, 18% of genes measured in a 907-spot microarray were expressed differently by at least 1.5 fold among individuals (Oleksiak *et al.*, 2002). Also in inbred strains of *Drosophila melanogaster* (Jin *et al.*, 2001) and clonal strains of yeast (Brem *et al.*, 2002), one quarter of their genes for basic cellular functions had significantly different gene expression. Therefore, care must be taken to ensure that the interpretation of results from gene expression studies is not simply due to natural variation between individuals or populations.

The field experiments in this study lasted for several months and the expression was relatively low. It was therefore necessary to confirm that the particular genes analysed could respond rapidly to stress and that their expression

could be maintained for an extended period. I did this by using a laboratory-based mesocosm experiment. This study tested the speed and duration of elevated gene expression to acute and chronic temperature challenge on mussel stress response. The particular genes chosen (*hsp24, hsp70, hsp90, elf2, tis11d*) had previously been shown to respond to environmental stress in mussels (Place *et al.*, 2008; Lockwood *et al.*, 2010). In my mesocosm study, genes responded quickly (within one hour) to acute thermal stress in the laboratory and the response was sustained for three weeks during chronic stress (**Figure 5.7**). This showed that the chosen target genes responded to stress and that changes in expression were sustained over long periods. Therefore, the low fold-changes in expression levels in the field experiments were unlikely to be an artefact of the experiment. Increased stress led to greater changes in expression of the target genes (**Figure 5.3**), which indicated that intensity of stress response could be assessed with these genes.

A challenge when studying stress response in the field is that only survivors can be measured. Those individuals that succumb to extreme or prevailing conditions are lost from the analyses. The use of mussels >3 cm in my field studies was meant to minimise the effects of predators on experimental mussels (Rilov and Schiel, 2006a). Therefore, it is probable that mussels experienced acute stress responses before perishing. Ideally, the stress responses of the weak mussels would be measured as they reached a critical threshold for survival. Unfortunately, detecting these types of responses is difficult in widely dispersed field sites, especially when conditions were so variable among them. One way of detecting critical thresholds is through tightly controlled laboratory experiments in which mussels are sampled throughout a period of thermal stress. A slow temperature increase and frequent sampling of individuals would be required to study the effects of temperature on dying animals. If they were sampled just prior to death, gene expression results could show the response changes of the weakest individuals. Expression of genes that mark the beginning of cell death such as apoptosis and specific damage such as microtubule depolymerisation, DNA damage and mitochondrial breakdown could be investigated as the physiological processes of stress-induced death.

Microarray and RNA-seq are better suited to field-based gene expression studies than is RT-qPCR, as they provide data about patterns of expression that vary between individuals which can then be investigated at the mechanistic level.

Microarray studies are a fertile area of investigation, which will have increasing use in future tests of stress responses. Transcriptomic studies are useful to investigate the response of the whole organism and can be useful to assess physiological response mechanisms of how environmental signals affect the organism.

There have been several transcriptome studies that have investigated the expression levels of mussels at temporal (Gracey *et al.*, 2008) and spatial scales (Place *et al.*, 2008; Place *et al.*, 2012) as well as between native and invasive species (Lockwood *et al.*, 2010). Until recently, large-scale microarray analyses were too costly and could have only been used to measure a limited number of samples. Studying the transcriptomic responses of mussels that are experiencing sublethal stress can provide insight into how the cellular machinery is affected by the stressors. During stressful situations, energy is often allocated away from growth or reproduction towards damage repair and prevention mechanisms (Hochachka and Somero, 2002); microarray data can be used to investigate the specific mechanisms that take place during this energy transition. Additionally, genes that are found to have expression patterns that are characteristic of specific conditions could be used as markers for the degree of stress and used in other systems.

6.4 Interactive effects of multiple stressors

Mesocosm studies have shown that multiple environmental variables can have interactive effects on an organism's fitness (Norkko *et al.*, 2005; Schneider *et al.*, 2010). For example, in one study, bivalves subjected to hypoxia and low food had strong decreases in scope for growth and in their RNA-to-DNA ratio, whereas bivalves subject to hypoxia alone had only slight decreases (Norkko *et al.*, 2005). Another study showed that mussels exposed to low food and high temperatures can have twice the mortality of mussels subjected to only high temperatures (Schneider *et al.*, 2010). The translocation study (**Chapter Three**) tested the effects of environmental conditions in different intertidal zones on multiple factors: coastal region, sites, animal size and species. Differences in stress response were seen at the site with the highest emersion time and temperature (Timaru), regardless of the species, size, intertidal zone or coastal region (**Figure 3.22 and 4.10**). Site-specific differences in physiological stress response are expected because sites by virtue of being in different geographical locations are different.

However, the absence of significant differences in the between-zone treatments was surprising. The conditions at Timaru are likely to have interacted to enhance the stress response at that site. Long-term studies have quantified water quality and site characteristics such as recruitment and species composition of all the sites used in these experiments except at Timaru (TIM) (Menge *et al.*, 1999; Menge *et al.*, 2003; Menge *et al.*, 2007; Bracken *et al.*, 2012). Food availability at Timaru is likely to be low due to the direction of ocean currents around the South Island (Knox, 1963). Box Thumb (BT) and Cave Rock (CR) have higher nutrients due to the back eddy north of Banks Peninsula, trapping and concentrating nutrients (Knox, 1963; Menge *et al.*, 2003; Schiel, 2004). Inputs from the Avon-Heathcote estuary also provide nutrients to Cave Rock and possibly Box Thumb (Environment Canterbury Regional Council, 2010). Thermal stress at Box Thumb was the lowest of all four sites, whereas thermal stress at Timaru was highest. Air exposure at Woodpecker Bay on the west coast was similar to Timaru but food was not limiting (Menge *et al.*, 1999; Menge *et al.*, 2003; Bracken *et al.*, 2012) and the mussel stress response was negligible. The interaction of low food and thermal stress amplify stress response. This could put future populations at risk due to decreased ocean productivity and higher temperatures as a result of global climate change.

In hindsight it would have been ideal to have undertaken water quality measurements to provide additional food availability information. This would have allowed for site-specific comparisons at the time of the experiment. I propose to analyse food availability using satellite data for chlorophyll-a and sea surface temperature in a fashion similar to that employed by Núñez-Acuña *et al.* (2012) during manuscript preparation of this data for publication.

6.5 Adaptive ability of mussel populations to long-term stress

The effects of global climate change on populations is likely to alter their biogeographic distribution (Bijlsma and Loeschcke, 2005) and understanding the physiological response to stress that underlies phenotypic plasticity and genetic fixation is essential (Visser, 2008). The translocation (**Chapter Three**) and transplant (**Chapter Four**) experiments were useful for investigating these concepts as they were designed to test the effects of changing environmental stress levels in a real-world scenario. In the transplant experiment, mussels from one source population were moved from their native site, which has a large mussel

population to sites with few mussels and left to acclimate over several months at their new site. In the transplant study, the highest stress response (*hsp70* gene expression) was seen after four months at the high stress site (Raramai), despite the time to acclimate. Substantial increases in *hsp70* expression indicating sublethal stress may have been accumulating. Little growth (**Figure 4.9**) and elevated *hsp70* expression (**Figure 4.10**) at the high stress site (Raramai) suggests the population might not have been sustainable.

Global climate change is a gradual process, yet the rate of climate change is high (IPCC, 2007). The limits of biochemical mechanisms studied through short-term physiological experiments can provide an indication of their capacity to shift in response to environmental conditions. These decadal changes will consist of the acclimatisation required to overcome the changing environment (Hofmann and Todgham, 2010). Organisms can relocate, adapt phenotypically or adapt genotypically over many generations to cope with climate change (Gienapp *et al.*, 2008). In the study from **Chapter Four**, the mussels were translocated to sites where they do not naturally occur in high abundance to test their response to more challenging conditions. The specific cause of low adult population could be due to stressful environmental conditions, recruitment failure, predation or other factors. However, the transplant sites are known to be more physiologically stressful than the source site (Menge *et al.*, 2003; Bracken *et al.*, 2012). There was limited physiological variability in the population to adapt to changes in environmental stress over the course of one summer, as seen in **Chapter Four** (**Figure 4.10**). Furthermore, in the present studies, gene expression could only be measured for animals that survived. Thus, having only data for the more resilient, surviving individuals, could under-estimate the severity of heat responses across a population. Therefore, the adult population may be more at risk to the effects of climate change than the data in **Chapter Four** suggest. Additionally, future populations of adult mussels will be dependent on the traits selected for during the settlement of larvae and early growth of juveniles

6.6 Zonation in New Zealand mussel beds

This study investigated some of the physiological responses that underlie zonation patterns by manipulating the shore height and location of mussels. *P. canaliculus* was predicted to be less tolerant to stress than *M. galloprovincialis*

because it inhabits lower regions in the intertidal zone (Menge *et al.*, 1999; Morton, 2004; Menge *et al.*, 2007). *P. canaliculus* was more susceptible at Box Thumb to extreme heat events than was *M. galloprovincialis*, as shown by heat-related mortality events (Petes *et al.*, 2007). However, the growth, survival and *hsp70* gene expression results from my study show that *P. canaliculus* generally responded better to stress than *M. galloprovincialis*. *P. canaliculus* survival was somewhat better than *M. galloprovincialis* (**Figures 3.8** and **3.9**). *Hsp70* gene expression in *M. galloprovincialis* was repressed at TIM, the most stressful site, suggesting that the animals were near death (**Figure 3.16**) (Frese *et al.*, 2003). Decreased *hsp70* expression has been linked to increased cell death (apoptosis) (Frese *et al.*, 2003), while increased *hsp70* has been linked to anti-apoptotic genes (Gracey *et al.*, 2008). This means the ecological data and the gene expression data are consistent for *M. galloprovincialis* at Timaru. *P. canaliculus* at Timaru have North Island-specific genetic haplotypes (Wood *et al.*, 2007) but the phylogeographic structure of *M. galloprovincialis* at Timaru and around the South Island is unknown. With northern haplotypes, *P. canaliculus* may be more physiologically adapted to warmer conditions than *M. galloprovincialis* at Timaru. Future study of the genetic population structure of *M. galloprovincialis* and *P. canaliculus* in the South Island would be important to investigate further the different responses seen at Timaru. Next generation sequencing technology would be able to better compare the stress response of the two mussel species as the expression of many more genes could be analysed.

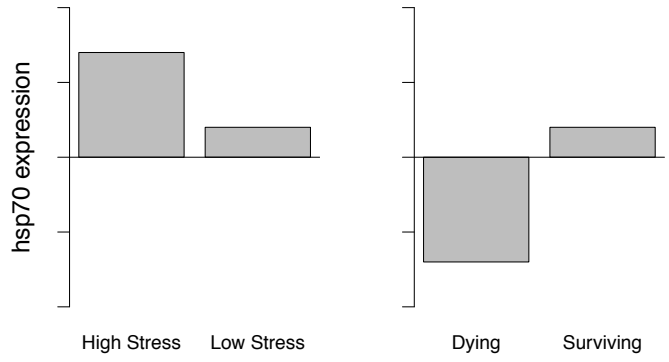


Figure 6.1 Model of *hsp70* expression patterns that indicate intensity of stress. These expression patterns would be relative to a control sample whose expression would constitute zero change.

Zonation can result from several mechanisms: differing physiological tolerances, predation, and interspecific competition. Menge *et al.* (2007) investigated differences in physiological tolerances between *P. canaliculus* and *M. galloprovincialis.* Inducible Hsp70 levels were measured at different shore heights in *M. galloprovincialis* and *P. canaliculus* that occupied the mixing zone of the two species. Higher on the shore, the animals of both species were found to have higher Hsp70 expression (Menge *et al.,* 2007) indicating that they were experiencing physiological stress. Higher on the shore, *M. galloprovincialis* Hsp70 expression surpassed that of *P. canaliculus* (Menge *et al.,* 2007). Menge *et al.* proposed that higher Hsp70 meant the animals were coping better to elevated stress levels. However, several studies (Anestis *et al.*, 2007; Place *et al.*, 2008; Dutton and Hofmann, 2009; Núñez-Acuña *et al.*, 2012) conclude that higher *hsp70* expression indicates the animals are more stressed. Dutton *et al.* (2009) also found that mussels are more susceptible to stress if they had higher *hsp70*.

6.7 Molecular tools in an ecological framework and wider applications of outcomes

The ability to investigate cellular processes in an ecological context has been augmented through the adaptation of molecular tools to the study of non-model organisms. The experiments in this thesis have brought together molecular techniques with classical ecological principles. Technology has advanced recently to allow molecular examination of non-model organisms. There have been only a few studies that describe a 'snapshot' of natural gene expression in mussels and link it to stress (e.g., during a tidal cycle (Gracey *et al.*, 2008) or at different locations (Place *et al.*, 2008)). The present study manipulated the stress experienced by the mussels to investigate how the different genes, identified through the literature of descriptive work, respond to the altered conditions. The use of these genes as metrics of stress could also be applied to other habitats or organisms. For instance, analyses of stress response gene expression in mussels from various aquaculture farms could provide insight to the fitness of organisms from different farms. Alternatively, they could be an advanced screening technique to guide farm placement based on sites with low mussel stress levels. Information about mussel fitness could direct the development of the Greenshell™ mussel aquaculture industry by aiding in the placement of new or expanding farms.

Additionally, the measures of stress used here could be applied to other organisms to assess how different species or populations are coping with the variable conditions associated with climate change. Direct measures of stress in natural populations, such as the targeted gene expression developed here, could help in the guidance of conservation efforts that would allow resources to be focused on more resilient populations than others which may be more vulnerable and unlikely to succeed.

6.8 Future directions

The data and results in this thesis provide a good foundation for additional development of molecular tools that can be used in ecological studies. Through careful optimisation, the genes used in this study could be applied as integrative biomarkers for environmental stress in a variety of systems.

There is little by way of phylogenetic detail for natural populations of mussels in New Zealand. A few studies have characterised the population structure of *M. galloprovincialis* and *P. canaliculus*, but the focus has been on populations in the North Island and the divide between islands at Cook Straight (Apte and Gardner, 2002; Star *et al.*, 2003; Wood *et al.*, 2007; Gardner and Westfall, 2012). Further study on the population structure around the South Island would greatly benefit the understanding of stress responses in mussel populations around New Zealand.

Additional mesocosm studies could be employed to investigate which stress factors may be most important for driving stress responses and to find critical thresholds that impact survival. Manipulations of individual and combined temperature, CO_2 levels, salinity, food and aerial exposure time could be used. Resulting gene expression levels could be measured with next generation sequencing (NGS) technology to study which biological processes are affected by the stressful conditions in the mesocosm.

The use of field manipulation studies to investigate stress response is not frequently done, as there are a multitude of factors that can affect the experiment in an uncontrolled manner. Drawing definitive conclusions from field studies of this nature can be challenging with RT-qPCR techniques. However, it is becoming easier to use increasingly complex molecular techniques to answer ecological questions with the rapid development and decreases in cost for NGS techniques.

Molecular ecology is paramount to understanding the physiological stress response. The use of NGS technology would potentially help to elucidate the physiological stress response of mussels in the New Zealand intertidal zone. Through the work of Menge *et al*. (2007) and myself, further study of the zonation patterns in New Zealand mussels is warranted. Future studies should be undertaken at a larger number of sites around the whole of New Zealand. Due to the variability between populations, many sites are required for regional comparisons. It would also be worthwhile to do species comparisons of stress responses between zones around the whole of New Zealand. Additionally, mussels could be transplanted from the South Island to the North Island and vice versa. Experimental studies that cover large geographical areas provide a better understanding of the true responses of species (Menge and Sanford, 2013).

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Appendix A: Gene Sequences

A.1. Hsp24

```
hsp24_sequence    ------------------------------------------------------------   60
     JF803805.1   GTGGGTGGGAATTAAACGAAATCAACAATACAGACGACTTTTAGCAGAACAGAGTAAAGA
       MGC00301   ------------TAAACGATATCAACAATACAGACGACTTTTAGCAGAACAGAGTAAAGA

hsp24_sequence    ------------------TTTGGAACCGCTACAATCAGTCTACACAGCATGATTTTGATG  120
     JF803805.1   TGACGTTTGTCCCAGTGCTTTGGAACCGCTACAATCAGTCTACACAGCATGATTTTGATG
       MGC00301   TGACGTTTGTCCCAGTGCTTTGGAACCGCTACAATCAGTCTACACAGCATGATTTTGATG
                              ****************************************

hsp24_sequence    ACATGTTTCACTTTATGGATGACTGGGAGCCTATGTCCGTAGGTTATGGGTATGGTCGTC  180
     JF803805.1   ACATGTTTCACTTTATGGATGACTGGGAGCCTATGTCCGTAGGTTATGGGTATGGTCGTC
       MGC00301   ACATGTTTCACTTTATGGATGACTGGGAGCCTATGTCCGTAGGTTATGGGTATGGTCGTC
                  ************************************************************

hsp24_sequence    A-----------------------------------------------------------  240
     JF803805.1   ACCCACCATGCCCGGGAATGGCAGTGCGTAGGAGGCGCAGACCGGAGACTTCACTGGCGG
       MGC00301   ACCCACCATGCCCGGGAATGGCAGTGCGTAGGAGGCGCAGACCGGAGACTTCACTGGCGG
                  *

hsp24_sequence    ------------------------------------------------------------  300
     JF803805.1   ATAAAAAGTGGACATACAGCGTAAAAATCGGAGACTTTGATGCACAGCATGTCAAAGTGA
       MGC00301   ATAAAAAGTGGACATACAGCGTAAAAATCGGAGACTTTGATGCACAGCATGTCAAAGTGA

hsp24_sequence    -
     JF803805.1   …
```

MGC00301 …

A.2. *Hsp70 M. galloprovincialis*

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AJ585375.1       1261 GATATATAACAGAAATAACTATATAGATATATTATCTTTCTAGATTATAAATAACTACTG 1320
hsp70_sequence        ------------------------------------------------------------

AJ585375.1       1321 AGATATGGCAGGAAAGGGTCCAGCAATCGGAATTGATTTAGGAACAACATACTCTTGTGT 1380
hsp70_sequence      1 ---------AGGAAAGGGTCCAGCAATCGGAATTGATTTGGGAACAACATATTCTTGTGT   51
                          ******************************-**********-********

AJ585375.1       1381 TGGTGTTTTCCAGCATGGAAAAGTAGACATCATAGCCAACGACCAGGGTAACAGAACAAC 1440
hsp70_sequence     52 TGGTGTTTTCCAGCATGGAAAAGTAGACATCATAGCCAACGACCAGGGGAACAGAACAAC  111
                       ***********************************************-**********

AJ585375.1       1441 TCCAAGTTATGTGGCTTTCACGGATACTGAAAGACTGGTTGGTGATGCAGCTAAAAATCA 1500
hsp70_sequence    112 TCCTAGTTATGTGGCTTTCACGGATACTGAAAGACTGGTTGGAAATGCAGCTAAAAATCA  171
                       ***-*************************************__****************

AJ585375.1       1501 AGTCGCATTGAACGCTACAAATACAATATTCGATGCCAAGAGACTGATCGGCAGAAATTT 1560
hsp70_sequence    172 AGTCGCATTGAACGCTACAAA-----------------------------------------  192
                       ********************

AJ585375.1       1561 TAGTGATTCGACAGTTCAGTCAGACATAAAACATTGGCCATTCAAAGTCATCAACAGCGG 1620
hsp70_sequence        ------------------------------------------------------------

AJ585375.1            …
hsp70_sequence
```

A.3. Hsp70 P. canaliculus

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hsp70_p sequence          ------------------------------------------------------------
MGC01310               327 CGGTCCTTCTGTTTCTCGTCTTCTGCCTTGTATTTCTCAGCATCATTGACCATGCGTTCA 268
hsp70_p sequence         1  -----TTCCTTGCTTATTCGTCCTTTGTCATTAGTGATACTAATCTTGTTCTCTTTGACT  55
MGC01310               267 ATTTCTTCTTTGCTCAAACGACCTTTGTCGTTAGTGATGGTGATTTTGTTCTCTTTACCA 208
                               *** ***** *  ** ******** ********  * ** ***********  *

hsp70_p sequence          56 GTACTCTTGTCCACAACTGATACATTGAAAATACCGTTTGTATCAATATCAAAAGTGACT 115
MGC01310               207 GTACTCTTATCTACTGCAGATACATTCAGGATACCATTGGCATCAATGTCAAAGGTCACT 148
                           ******** ** **  * ******** *  ***** ** * ****** ***** ** ***

hsp70_p sequence       116 TCAACCTGTGGCACACCTCTTGGTGCTGGTGGGATTCCAGTCAAATCAA----------- 164
MGC01310               147 TCAATCTGGGGCACACCTCTTGGTGCTGGAGGTATTCCAGTTAATTCAACCATTCCAACA  88
                           **** *** ******************** ** ******** ** ****

hsp70_p sequence          ------------------------------------------------------------
MGC01310                87 AAACAGACACAAACCTTCACTACCTACTCTGACAATCAGCCTGGTGTATTAATCCAGGTT  28
hsp70_p sequence          ---------------------------
MGC01310                27 TATGAAGGAGAGAGAGCTATGACCAAG   1
```

A.4. Hsp90

```
AJ586906.3        2701 AACTGGACCTTGGAAAAGATCTGGAAATCAGAATCATACCAGACAAGGATAACAACACAC  2760
hsp90 _sequence       ------------------------------------------------------------
AJ586906.3        2761 TAACCATCATTGATACTGGTATTGGAATGACCAAAGCTGATCTGGTCAATAACCTGGGTA  2820
hsp90 _sequence       ------------------------------------------------------------
AJ586906.3        2821 CC  2822
hsp90 _sequence        --

AJ586906.3        2823 ATTGCCAAGTCTGGTACTAAAGCTTTCATGGAGGCTCTTCAAGCTGGAGCTGATATTTCT  2882
hsp90 _sequence    108 TTTGCCAAGTCTGGTACTAAAGCTTTCATGGAGGCTCTTCAAGCTGGAGCTGATATTTCT    49
                       -************************************************************

AJ586906.3        2883 ATGATTGGACAGTTTGGTGTAGGTTTCTACTCCGCCTACCTGGTTGCT  2930
hsp90 _sequence     48 ATGATTGGACAGTTTGGTGTAGGTTTCTACTCCGCCTACCTGGTTGCT     1
                       *********************************************

AJ586906.3        2931 GATAAAGTAGTTGTCCAGACAAGGAACAACGATGATGAGGAATATATCTGGGAATCAGCA  2990
hsp90 _sequence       ------------------------------------------------------------
AJ586906.3        2991 GCTGGGGGATCATTTACAGTCAAAACAGTATCAGGTAAAGAGAGTTGATAATTGATGGGG  3050
hsp90 _sequence       ------------------------------------------------------------
```

A.5. Elf2

```
elf2_sequence            ------------------------------------------------------------
MGC04450          961 GTTTGACTCTTTTATATAAAAATATACAAAAAAATAAATATCAAATATAAACAATCTATA 902
elf2_sequence            ------------------------------------------------------------
MGC04450          901 AATTATGAGATTTTTACAACAATATAACAATAATAGGTGGATTATCAACTCCTTTGTAGA 842
elf2_sequence       1 ------------GCTACATGCTAACGGAGAACCTAATTCCCTCTACAGAGATACAACACT  48
MGC04450          841 ATACAATAATAGGCTACATGCTAACGGAGAACCTAATTCCCTCTACAGAGATACAACACT 782
                          ********************************************

elf2_sequence        49 GACAATCTTAACTAGATTATCAACTCCAATACACCACGTGACTTCAGTCGGATTTCTGAT 108
MGC04450          781 GACAATCTTAACTAGATTATCAACTCCAATACACCACGTGACTTCAGTCGGATTTCTGAT 722
                         ************************************************************

elf2_sequence       109 TCGATAGAACCTTTACGATGGCCAATGTCGGATTTCAGATTTTATAGAAAATGATTGTCA 168
MGC04450          721 TCGATAGAACCTTTACGATGGCCAATGTCGGATTTCAGATTTTATAGAAAATGATTGTCA 662
                         ************************************************************

elf2_sequence       169 TGTATGATTTGAGTCACGAAATGAGGAGTGGCCATACACCATTACTTGGTTGCTGATTTA 228
MGC04450          661 TGTATGATTTGAGTCACGAAATGAGGAGTGGCCATACACCATTACTTGGTTGCTGATTTA 602
                         ************************************************************

elf2_sequence       229 TTTCTATTCCTCTAATACTGATGTTTTGAGATTGTCCTTGGGCGGAATCTACCGAGCTCA 288
MGC04450          601 TTTCTATTCCTCTAATACTGATGTTTTGAGATTGTCCTTGGGCGGAATCTACCGAGCTCA 542
                         ************************************************************

elf2_sequence       289 ACTCTCCGAGATCTTTTTGGTATTTCTCGTCATTCATCGTCATCTTGCGG---------- 338
MGC04450          541 ACTCTCCGAGATCTTTTTGGTATTTCTCGTCATTCATCGTCATCTTTCGGAATTAACTCA 482
                         ******************************************_***
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elf2_sequence            ------------------------------------------------------------
MGC04450            481 AGCGGTTAACTAAGACTGGCAACCTAATGTTCTCATTCTTTCCAGCCAGTGGAAAATGGT 422

elf2_sequence            ------------------------------------------------------------
MGC04450            421 CCAAATTTGTAAACCAGTCGTCTACCGTCGACTCGTTCGAGAATGCCTCGTTTGTAATAG 362
```

A.6. Tis11d

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tis11d_sequence     --------TGTATGGAATGTACGACAGAGTTCTGTTTTATACTTGGGATGACGATTCAAT 52
MCG01771            AATCCAATAGTATGGAATGTACGGCAAAGTTCTGTTTTATACTTGGGATGACGATTCAAT 60
                           :***************.**.*********************************

tis11d_sequence     GTACGAAGTTCATGACCACCGTGAGCAAACTGACACTTATCCCCGTATTTACAATGTCCA 112
MCG01771            GTACGAAG-TCATGTCCACCGTGAGCAAACTGACACTTATCCCCATATTTACAATGACCA 119
                    ******** *****:*****************************.***********:***

tis11d_sequence     CTTTCCTCGAAGGGTCGGCACAATTCTGTTTTGTAACGACTGGAGTTAACATTATTCCTT 172
MCG01771            CTTTCCTCGAAAGGTCGGCACAATTCTGTTTTGTAACGGCTCGAGTTAACATTATTCCTC 179
                    ***********.**************************.** *****************

tis11d_sequence     TGAATACTCAAACTGCTCAAAGCTCGGTCACCGATCGGTTCACTTATACTCCTATCCAAC 232
MCG01771            TGAATACTCAAACTGTTCAAAGCTCGGTCACCGATCGGTTCACTTATACTCCTATCCAAT 239
                    *************** ****************************************

tis11d_sequence     TTGCGATGTAAATTCTCATTCT--GATTAAGATCAGTTACAC-CATTCTGTTGTGTAATT 289
MCG01771            TTACATCCAAAATGCAAAGTATTGGACGAAATGCGGACTCTTTCAATAGCTAGCACATTT 299
                    **.*.:  :**** *:.* *.*  **  **.: *.*: :*:  **:*.  *:* . *:**

tis11d_sequence     GTAATGAAAGAACTTGAAGACATATTTCCATTTCTAGACATCATAGCACCAATTGTAGCC 349
MCG01771            GTATAAAAACCCGTTTCTTGGGTTGTACAAAAACATAATACTATTATACTCAAC-AAGAA 358
                    ***::.*** .. ** .: . .*: *:*.*:::*::.* *  **:. ** .*:  :**..

tis11d_sequence     CTTCTTTCATTGAACTGTTTCATCTGCTGGTTTCTGCGATTCTGT 394
MCG01771            CTGGAATTATT-AACT-TTAAAAATGCCC-TATCAACATGAAGG- 399
                    **  ::* *** **** **:.*:.***   *:**:.*.: :. *
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